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# BIOLOGICAL REVIEWS

*of the*

*Cambridge Philosophical Society*

Edited
by
H. MUNRO FOX

VOLUME 15

CAMBRIDGE
AT THE UNIVERSITY PRESS
1940

PRINTED IN GREAT BRITAIN

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# THE ANTIHORMONES

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(*Received* 12 *March* 1939)

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## I. HISTORY[1]

The idea of the production of a hormone-inhibitory substance which circulates in the blood stream and is able to neutralize the effects of a hormone was probably first expressed by Möbius (1906). He found that the blood of thyroidectomized sheep neutralizes the action of thyroid hormone. Preparations of the blood of such thyroidectomized animals have been made available by the Merck factory under the name of "Antithyreoidin Möbius". Several authors claimed to have obtained good results with this preparation in cases of Graves's disease, but since Sonne (1914) was unable to obtain any effect in animal experiments and found normal blood to be just as effective in patients as the blood of thyroidectomized animals, he claimed the results to be merely psychic. Pychlau (1913) states that the milk of a thyroidectomized woman exerted a curative effect in such a case.

The first author who tried to obtain a hormone-neutralizing substance using an endocrine preparation as an "antigen" was probably Blum. Later he repeated Möbius's experiments and confirmed the findings on thyroidectomized animals, but at the same time he noted that thyroid hormone-inhibitory substances are also present in the blood of normal sheep. The name "catechin" was given to these inhibitory substances (Blum, 1933). Schäfer (1924) termed inhibitory hormones "chalones". Blum's experiments were later extended by Legiardi-Laura (1919, 1923, 1934) and Legiardi-Laura & Brim (1929), who treated horses with posterior pituitary preparations and claimed that the serum of such animals cures the glucosuria in many cases of diabetes. This serum has also been stated to be effective in the treatment of hypertension (Legiardi-Laura & Brim, 1929). The experiments of these authors and of Blum are difficult to evaluate, since little is said in their original publications about the nature of the antigen used, the doses given, or other details concerning the treatment of the donor animal. The experiments of Koyano (1923) are open to the same criticism. He injected beef pituitary emulsions intraperitoneally into male rabbits, and stated that the serum of such animals produces marked histological changes in the hypophysis of rats. He called his preparation "a specific immune serum".

In 1924 Cotte published a series of experiments in which extracts of fowl ovaries were chronically injected into male rabbits. Thus a "sérum anti-ovaire" was obtained. This serum changed the colour of the plumage in hen-feathered cocks. The experiment was interpreted as a further proof of Morgan's theory concerning the "corpus luteum cell nature" of certain cells in the testes of the hen-feathered cock.

In the same year de Jongh observed that certain insulin preparations are less active in larger doses than in small ones, while very large amounts of the same preparation again show an increase in potency. Mathematical considerations led the author to interpret this double-peaked curve as the result of an insulin-inhibitory substance superimposed on the usual insulin effect. The name "anti-insulin" was coined for this substance. Further purification removed this "anti-

[1] Antihormones have already been reviewed by Collip (1934, 1936) and Guyénot *et al.* (1937).

insulin" from the original preparation. Similar experiments led Nobel & Priesel (1925) to the assumption that anti-insulin is present in crude pancreatic extracts. Meyer-Bisch *et al.* (1928) observed that pancreatic secretion increases the blood sugar of experimental animals and concluded that anti-insulin is excreted into the duodenum. The primary hyperglycaemia observed after the administration of crude insulin preparations has also been attributed to the presence of anti-insulin (Wichels & Lauber, 1932). Sahyun & Blatherwick (1928) observed that rabbits on a high carbohydrate diet, injected chronically with insulin, became "immune" to this hormone, so that even 70–100 units did not elicit convulsions. The blood-sugar response in such "immunized" animals was also much lower than in the non-pretreated controls.

The name "antihormone" was also used by Wiese (1928). This author found that testicular extract coagulates the follicular fluid of nymphomanic cows, but has no effect on the follicular fluid of normal animals. This phenomenon was attributed to the formation of "antihormones" in the nymphomanic cow. The administration of testis extracts was found to be effective in increasing ovarian activity in cattle, and the author believed this to be due to the production of "antihormones".

The earlier experiments of Blum on "catechin" aroused particular interest because of the good clinical results which many authors claimed to have obtained with these preparations. Numerous publications have appeared more recently on similar antithyroid hormone preparations. Thus Herzfeld & Frieder (1933) have prepared a catechin from blood, and this preparation is now on the market under the name of "tyronorman". It is said to alleviate the symptoms of Graves's disease. It is interesting to note in this connexion that Branovacky (1926) showed that the blood of patients suffering from Graves's disease increased the oxygen sensitivity of rats in the well-known Asher experiment, and that this effect was neutralized by the administration of blood serum from myxoedema patients. Asimoff (1926*a*, *b*) reported that the serum of thyroidectomized sheep inhibits the metamorphosis effect of thyroid hormone in the axolotl and Gürber & Geszner (1928) found that this metamorphosis-antagonizing principle is in the euglobulin fraction. Since, however, Lewit *et al.* (1930) obtained similar effects with normal blood, they doubt the specific nature of such catechins. Recently, Saegesser (1933) observed that the serum of myxoedema patients neutralizes thyroxin in Reid-Hunt's acetonitril test—a finding which reminds one of Branovacky's experiments concerning the decrease in oxygen sensitivity which we discussed above. Saegesser stated that his antithyroid substance was lipid-soluble. Since he found that cholesterol exerts a similar effect, he considered it possible, however, that this action was a simple chemical antagonism.

Rzentkowski (1912) claimed to have obtained complement deviation with the serum of hyperthyroid patients using thyroid extract as an antigen. This report on the historical development of our ideas on the production of hormone-antagonizing substances would not be complete without mentioning that thyroglobulin-antagonizing substances were obtained by the administration of thyroglobulin by

numerous workers (Hektoen *et al.* 1923, 1927; Hicks, 1926; Schulhof, 1930), and that specific antiferments have been produced and demonstrated in the blood of experimental animals chronically treated with extracts of endocrine glands by Abderhalden (1918) who termed such ferments "Abwehrfermente".

The "cytolysines", which are immune substances formed against the protein of injected cells, have nothing to do with antihormones. It is not justified, therefore, for Del Solar (1938) to speak of "antihormones" if a testis cytolysive is formed under the influence of continued testis extract treatment.

Reiss *et al.* (1931*a*, *b*, *c*) found that the gonadotrophic effect of pregnancy urine preparation may be inhibited by alkaline aqueous beef pituitary extracts, both in the immature male and female rat. Since their extract proved to be free of growth hormone, they inferred that the hypophysis contained a principle capable of antagonizing the gonadotrophic hormone, and that this principle was not identical with the growth hormone. These findings have been confirmed and extended by Evans *et al.* (1936), who termed the principle in question the pituitary "antagonist".

It became known, furthermore, that upon continued administration the action of certain hormones decreases considerably. Thus, for instance, the effect of the thyrotrophic hormone on the guinea-pig thyroid vanishes after a certain time, even though daily injections of an initially stimulating dose are continued (Aron, 1930; Loeb & Friedman, 1931), and the same is true of the gonadotrophic action of pregnancy urine preparations in the mouse (Zondek, 1931) and rat (Collip, 1932; McPhail, 1933).

The fact that this phenomenon is actually due to a specific immunity to the gonadotrophic principle and not simply to an exhaustion of the ovary was then demonstrated by Selye *et al.* (1934*b*), who showed that rats rendered insensitive with a pregnancy urine preparation by continuous pretreatment still reacted with intense ovarian enlargement when treated with a pig pituitary gonadotrophic extract. Later, these same investigators (1934*c*) showed that rats may also become insensitive to daily rat pituitary implants, and that the ovaries of such animals maintain their sensitivity to the gonadotrophic action of human pregnancy urine extracts.

The first demonstration of the appearance of hormone antagonists in the blood as a result of pretreatment with a hormone preparation was published by Collip & Anderson (1934) and Anderson & Collip (1934) for the thyrotrophic, and by Selye *et al.* (1934) for the gonadotrophic hormone. It was mainly on the basis of these observations that Collip (1934) formulated his antihormone theory, according to which the action of certain hormones is antagonized by others, the specific physiological function of which is to neutralize the action of hormones. Concerning their nature he stated that "there is evidence that they are not comparable to antibodies in the sense usual in immunology" (Collip, 1935*b*).

## II. THE ANTIGONADOTROPHIC HORMONE

### (1) *Chemistry*

According to Harington & Rowlands (1937), the antigonadotrophic activity of the antiserum to the gonadotrophic serum of human pregnancy urine prepared in the goat can be quantitatively recovered from the globulin fraction. There is an equal distribution between the pseudo- and euglobulin fractions. It might prove of interest for the purification of antihormone preparations that precipitation of the antibovine protein by adding bovine serum to an antiserum against a bovine pituitary gonadotrophic extract does not alter its antihormone titre so that at least part of the unwanted protein may be removed from these sera without loss of potency (Gegerson *et al.* 1936).

The precipitate of the antiserum obtained by pouring it into three times its volume of acetone is repeatedly washed by acetone and then readily preservable *in vacuo* for at least 6 months without noteworthy loss of antigonadotrophic effect. By this method, 1 c.c. of serum yields about 50–70 mg. of a readily water-soluble powder (Zondek & Sulman, 1937*b*).

### (2) *Bio-assay*

In the *mouse* Twombly & Ferguson (1934) and Twombly (1936) assayed the antigonadotrophic hormone by injecting 0·1 c.c. of the antiserum together with a known amount of gonadotrophic hormone (usually 1 mouse unit) for each of five injections over a period of 30 hr. The animals were killed, 100 hr. after the first injection, and the presence of corpora lutea and the ovarian weight recorded. Laroche & Simonnet (1936) availed themselves of a similar method using the male mouse as a test object.

In the *rabbit*, according to Bachman *et al.* (1934), the assay is performed on adolescent virgin does of over 1800 g. body weight. They are given a known amount of the gonadotrophic hormone four times intravenously at intervals of 12 hr., each such dose being preceded 2 hr. earlier by the injection of 1 c.c. of the antiserum. The animals are killed 24 hr. after the last injection and their ovaries examined for corpora haemorrhagica and apical corpora lutea. A similar method is also used by Moricard (1936).

In the *rat* the typical assay, according to Selye *et al.* (1934) and Bachman *et al.* (1934), is performed by pretreating 21-day-old females on 6 consecutive days with subcutaneous or intraperitoneal injections of 1 c.c. of antiserum daily. During the last 3 days, varying doses of gonadotrophic hormone are also given, while control animals receive only gonadotrophic preparations during the last 3 days. The potency of the antigonadotrophic serum is then determined by the number of rat units of gonadotrophic hormone which a given amount of serum is able to inactivate. Engel (1935*d*, *e*) used a similar method, except that he injected the immature test rats with 10 units of gonadotrophic hormone (divided into 6 subcutaneous doses) within 48 hr., together with the antihormone preparation. One hundred hours

later the animals were killed and their ovaries, uteri and vaginal smears were compared with those of controls not receiving antiserum. Brandt & Goldhammer (1936*a*) use 25–35 g. rats receiving 20 rat units of the gonadotrophic hormone divided into three injections within 24 hr. in combination with 0·5 c.c. of the antiserum. Autopsy follows after 100 hr., and the potency of the serum is determined by comparing the sex organs of these animals with those of controls not receiving antihormone but only the same amount of the gonadotrophic preparation. A very similar test has also been used by Zondek & Sulman (1937*b*), who define the antigonadotrophic unit as the smallest amount of the antiserum which is able to prevent the ovarian effect of 1 rat unit of the gonadotrophic hormone.

### (3) *Theories*

#### (*a*) *Specificity of the antigonadotrophic hormone.*

In the cases of antigonadotrophic hormones, just as in that of all other antihormones, one of the most debated problems is the specificity of its action. Here, again, we have to differentiate between (1) *species specificity*, that is to say, the question whether an antiserum obtained with a gonadotrophic hormone of a certain animal species can immunize against a gonadotrophic hormone of another species; (2) *organ specificity* (sometimes also referred to as "source specificity"), that is, whether a gonadotrophic hormone prepared from a certain organ, let us say, the hypophysis, can immunize against a similar preparation obtained from another organ of the same species, for instance, the placenta; (3) *extract specificity*, that is, whether an antiserum against an extract prepared by a certain method would necessarily immunize against gonadotrophic preparations made from this same source by different methods; (4) *hormone specificity*, that is, the question whether an antigonadotrophic serum obtained with the follicle-stimulating hormone could render animals resistant to the luteinizing hormone and vice versa.

Much work has been done in order to clarify these questions because they are of great importance for the elucidation of the most fundamental problem in connexion with the antihormones, that is, the question of their physiological significance. It should be emphasized, however, that in practice it is not very easy to keep these four questions regarding specificity strictly apart, because the relative proportion between the follicle-stimulating and the luteinizing ability of the hypophysis differs in various species, so that an apparent species specificity may be due to the fact that the animal became adapted to a different mixture of the two gonadotrophic principles. This same possibility must be considered in the case of experiments conducted to answer the question of organ or even extract specificity.

*Species specificity.* The first experiments concerning the question of species specificity were those of Selye *et al.* (1934*b, c*), who showed that rats rendered resistant against the gonadotrophic action of rat pituitary implants were still responsive to pregnancy urine extracts and vice versa. Later, Fluhmann (1935*b*) prepared an antiserum to an extract of human pregnancy serum, which failed to inhibit sheep pituitary extract, but since it also failed to inhibit human pituitary

extracts, his experiment shows organ specificity and consequently gives no clue concerning the question of species specificity. Gustus *et al.* (1935) prepared an antiserum against a gonadotrophic extract of pregnant mare serum in the monkey. They found that the serum inhibited the action of the extract with which it was prepared, but had no noteworthy neutralizing effect on human pregnancy serum or preparations of sheep or human hypophyses. Brandt & Goldhammer (1936*a*) claimed that an antiserum against the gonadotrophic principle of pregnancy urine inhibits the gonadotrophic action of human placenta, pituitary, and pregnancy serum extracts, but does not antagonize gonadotrophic preparations made from pregnant mare serum. Gegerson *et al.* (1936) claimed that an antiserum obtained by treatment with human pregnancy urine antagonizes the gonadotrophic effect both of this same preparation and of cattle pituitary extracts, but an antiserum produced by injecting the latter did not inhibit the action of the pregnancy urine extract. Since, on the other hand, both these antisera inhibited the gonadotrophic effect of pregnant mare serum, one can hardly regard these experiments in favour of species specificity.

Kabak (1936) found that in rats rendered insensitive to pregnancy urine extract a positive gonadotrophic effect was still attainable by implanting rat or rabbit pituitaries or injecting sheep pituitary extracts or pregnant mare serum. Since, on the other hand, these animals failed to respond to human pituitary extracts, the author concluded that the immunity obtained is species-specific but not organ-specific.

Thompson & Cushing (1937) found that an antiserum prepared by injecting a dog with pregnant mare serum inhibited the gonadotrophic activity of sheep or pig pituitary extract, but had only a slight neutralizing effect on the action of human pregnancy urine extracts. The serum of dogs immunized with sheep pituitary, on the other hand, was quite active in antagonizing the action of pig pituitary, human pregnancy urine, human menopause serum and pregnant mare serum. They concluded that there is no strict species specificity. Collip (1937) showed that an antiserum obtained from sheep chronically treated with a sheep pituitary extract inhibited the gonadotrophic effect both of this same extract and of pregnant mare serum, although it had no effect on the action of a pregnancy urine preparation. Zondek & Sulman (1937*a*, *b*) and Sulman (1937) prepared immune sera against human pregnancy urine and pregnant mare serum and studied their ability to antagonize the effect of these same preparations, cattle pituitary, human pregnancy blood and human pituitary gonadotrophic preparations. They concluded that there is some organ specificity and a high degree of species specificity. They emphasize, however, that a trace of inhibition is observed with any antigonadotrophic serum against every other gonadotrophic extract which they tested. Parkes & Rowlands (1936) found that an antigonadotrophic serum prepared by prolonged injection of rabbits with an ox pituitary extract inhibited the ovulation otherwise produced by the antigenic extract itself or a similar extract of horse pituitary or a saline suspension of fresh cow and sheep hypophyses. This serum, given intravenously to rabbits immediately after mating, inhibited the ovulation which would normally occur

within 10–12 hr. The latter effect has been regarded as a passive immunization against the gonadotrophic hormone secreted by the rabbit's own pituitary. The authors concluded that: "There is thus no suggestion of species specificity in the anti-ovulation action of this serum." Later, however, Parkes & Rowlands (1937) found that treatment with rabbit sera known to be capable of neutralizing thyrotrophic and gonadotrophic ox pituitary extracts, and gonadotrophic preparations of horse pituitary, pregnant mare serum and human pregnancy urine did not cause any plumage or comb changes suggestive of hypophyseal insufficiency in the fowl. They concluded: "that antisera to mammalian thyrotrophic or gonadotrophic substances are ineffective against avian pituitary substances and that there is a class specificity, if not a species specificity, in the immunological (haptene) character of the pituitary hormones". Recently Rowlands (1937, 1938*b*) made an extensive study of this question by preparing antisera against human pregnancy urine extract, horse pituitary, ox pituitary and pregnant mare urine extract, and testing their neutralizing effect against all of these gonadotrophic preparations. He concluded that: "Complete 'species-specificity' is shown by the reaction of the antisera to gonadotrophic extracts of human urine of pregnancy and pregnant mare serum, whereas antisera to similar extracts of pituitary origin exhibit only incomplete 'species-specificity'. Complete 'source-specificity', on the other hand, is only found in the reactions of the antiserum to gonadotrophic extracts of pregnant mare serum."

*Organ specificity*. Fluhmann (1935*a*) produced an antiserum to human pituitary extracts in the rat and found it to neutralize not only the effect of this same extract but also that of a gonadotrophic preparation made from human pregnancy serum. In a second paper, however, Fluhmann (1935*b*) reported the preparation of an antiserum to a human pregnancy blood extract which failed to inhibit the gonadotrophic effect of a human pituitary extract. Twombly (1936) obtained an antiserum against an extract prepared from the urine of a patient suffering from teratoma testis which inhibited the effect of pregnancy urine extracts, but this is not, strictly speaking, evidence for or against the organ specificity of these antisera. Brandt & Goldhammer (1936*a*) noted that antisera against human pregnancy urine antagonized the gonadotrophic effect of extracts from other tissues of the human body, such as the hypophysis, the placenta, or pregnancy serum. Kabak (1936) was unable to elicit any gonadotrophic action by means of human pituitary extracts in rats rendered insensitive to human pregnancy urine preparations, while the same rats remained sensitive to extracts of rat, rabbit, sheep or horse pituitaries. De Fremery & Scheygrond (1937*b*) also noted that antisera obtained by treatment with pregnancy urine extracts antagonize the action of human pituitary preparations; yet Zondek & Sulman (1937*a*) found that an antiserum obtained by treatment with human pregnancy urine neutralized this same extract more actively than it did preparations of human pregnancy blood or human pituitary gonadotrophic preparations. This organ specificity, however, was not absolute, for there was some inhibition even in experiments in which the antisera were tested against extracts prepared from sources other than the antigenic preparation. Rowlands (1938*b*)

found that an antiserum obtained in rabbits by treatment with a pregnant mares' serum preparation was the only one among the antisera he tested which was completely organ-specific, in that it neutralized the gonadotrophic hormone from mares' serum but had no effect on that from the horse's pituitary body.

*Extract specificity.* Clear-cut examples of extract specificity are as yet missing in the case of gonadotrophic antisera.

*Hormone specificity.* The fact that antigonadotrophic serum is specific in the sense that it does not inhibit the action of other hormones has already been emphasized by Collip (1934), but evidence for or against strict specificity between sera obtained against the follicle-stimulating and the luteinizing factor respectively is still lacking. Selye *et al.* (1934*b*, *c*) interpreted their finding that resistance against pregnancy urine extracts does not immunize the rat against rat pituitary implants and vice versa as showing that the gonadotrophic principle of the urine differs from that of the hypophysis. According to Guyénot *et al.* (1937), guinea-pigs form antihormones against the luteinizing principle of cattle hypophyses more readily than against the follicle-stimulating factor. Many of the findings quoted in the section on species and organ specificity may also be regarded as giving indirect evidence for or against the hormone-specific nature of these extracts, since it is known that some of the preparations there mentioned have a higher follicle-stimulating potency while others contain more of the luteinizing principle. This evidence is, however, necessarily inconclusive, as it is impossible to decide, in case the extracts are made from different sources, whether any specificity observed should be regarded as organ specificity, species specificity or hormone specificity. The only manner in which this question might be attacked would be to test antisera against a follicle-stimulating extract prepared from a certain source for their ability to antagonize the action of luteinizing preparations of the same source and vice versa. Even in this case, however, the question of extract specificity might arise.

### (*b*) *Nature of the antigonadotrophic hormones.*

The most discussed questions concerning the nature of this and other antihormones are: (1) the relationship of antihormones to true *serological antibodies* or *haptenes*; (2) the possible interpretation of antihormones as specific *protective ferments*, the function of which is to destroy the hormone; (3) the question whether these antihormones represent *physiological body constituents* or whether they are only found under abnormal conditions, such as exist when an organism is treated with injections of a hormone extract.

*The antibody theory.* (1) *Arguments against.* Collip (1934, 1935*a*) considers it very unlikely that antihormones should simply represent serological antibodies against hormones of protein nature because rats have been made resistant to the maturity hormone of rat pituitary by continued implantation of rat hypophyses (Selye *et al.* 1934*c*) and an antiserum against sheep pituitary gonadotrophic hormone has been obtained in sheep treated with a sheep hypophysis extract (Collip, 1937). It had been shown, furthermore, that the serum of certain human beings

contains a principle which inhibited the action of gonadotrophic preparations, although these patients were not pretreated with such hormone extracts (Collip, 1935*a*).

Bachman (1935) showed that there is no strict parallelism between the antihormone content of the serum of rabbits pretreated with pregnancy urine extracts and its content in true antibodies directed against the antigenic extract. This lack of parallelism has also been emphasized by Gustus *et al.* (1935), Eichbaum & Kindermann (1935), Kindermann & Eichbaum (1936), Brandt & Goldhammer (1936*a*), Demanche *et al.* (1937), and Sulman (1937), who, like Bachman, claimed that the antibodies produced by pregnancy urine extracts are not directed against the hormone itself, but against some non-specific "urine antigen". Gegerson *et al.* (1936) removed the antibovine protein present in their pituitary antiserum by treatment with normal bovine serum and showed that this does not remove the antihormone activity from the serum. They concluded that the antihormone effect "is not due to an antihuman protein phenomenon *per se* but to the presence of another antagonistic but specific substance". One of the strongest arguments against the interpretation of antihormones as antibodies is the demonstration by Parkes & Rowlands (1936) that an antigonadotrophic serum obtained by treatment of rabbits with an ox pituitary gonadotrophic extract may prevent ovulation after mating when injected into another rabbit, a fact which has been considered to result from the inhibition of the ovary-stimulating effect of the natural gonadotrophic hormone released from the rabbit's pituitary. More recently Kupperman *et al.* (1939) showed that an antiserum obtained from rabbits chronically treated with sheep pituitary extract inhibits the excessive luteinization of the ovary normally resulting from the endogenous gonadotrophic secretion of the castrate partner's pituitary in female-female or female-male parabiotic rats. The substance is effective when injected either into the normal or the gonadectomized partner. All the reports mentioned in previous sections in which an antigonadotrophic serum produced by a gonadotrophic extract of a certain species proved antagonistic to a similar extract prepared from tissues of another species might also be regarded as speaking against the true antibody nature of these antihormones.

(2) *Arguments in favour.* Contrary to the above mentioned investigators, Du Shane *et al.* (1935) and Martins (1935) showed that if a castrate female rat was parabiotically united with a normal or hypophysectomized female partner, the sex organs of the latter, and especially its ovaries, enlarged considerably under the influence of the excess gonadotrophic hormones secreted by the pituitary of the spayed parabiotic twin. This enlargement persisted for many months and no loss of sensitivity was demonstrable. The authors concluded that physiologically administered gonadotrophic hormones do not produce antihormones and regard the latter as serological antibodies formed against the partially denatured protein of the hormone extracts. Katzman *et al.* (1937) implanted rats with rat pituitaries daily over a period of up to 9 months, or injected them with alkaline extracts of rat pituitaries, without observing any signs of refractoriness. They also concluded that the antigonadotrophic hormone is an antibody which cannot be produced by

treatment with a homozoic extract. Similar findings have been reported by Artemov (1937).

Ehrlich (1934, 1935) showed that continued treatment with gonadotrophic extracts resulted in the formation of specific antibodies and the author expressed the opinion that the antihormones are identical with the complement-fixing antibodies which he obtained. Similarly, Twombly (1936) states that the antihormone content of the serum of rabbits treated with a gonadotrophic preparation of pregnancy urine runs closely parallel with its content in precipitins against the antigenic extract. Since hormone preparations partially inactivated by heat or completely inactivated by ageing have approximately the same ability to form protective antisera as active preparations of the hormone, Twombly is inclined to agree with the antibody theory. The fact that even very prolonged treatment with pregnancy urine extracts fails to produce antihormones in the human being and the observation that non-protein hormones (e.g. oestrin) do not form antihormones under any condition confirmed this author in his opinion. Fremery & Scheygrond (1937*a*) obtained similar results, inasmuch as they prepared a potent antigonadotrophic serum by injecting rabbits with a male urine extract which was almost completely devoid of gonadotrophic activity. They concluded that the urine contained substances having the same antigenic potency though not the same gonadotrophic effect as the principles present in pregnancy urine. Zondek *et al.* (1938), on the other hand, found that although urinary gonadotrophic preparations inactivated by boiling retain their antihormone-forming ability, this is merely due to traces of the hormone still present in these preparations. "After complete inactivation through heat, prolan is neither able to produce antiprolan *in vivo* nor 'paralyse' it *in vitro*."

*The haptene theory*. Sulman (1937) examined the question of whether or not the gonadotrophic hormone could act as a haptene. Haptenes are fully effective *in vitro*, but *in vivo* their immunizing ability is restricted since they can only develop antigenic properties in close connexion with a carrier substance. In this manner, the haptene, though native to the body, can act as if it were a foreign substance and can incite the production of antibodies. Sulman (1937) used pregnancy urine, in combination with pig serum as a carrier, and found that the hormone cannot act either as an antigen or as a haptene. Parkes & Rowlands (1937), however, favour the haptene theory as it may explain why the serum of rabbits immunized with ox pituitary extracts prevents post-coital ovulation in other rabbits.

*The protective ferment theory*. Sulman (1937) and Zondek & Sulman (1937*a*) came to the conclusion that since the gonadotrophic hormone does not act as an antigen or a haptene, it must necessarily be a protective ferment or "Abwehrferment" in the sense in which Abderhalden uses this term, but their conclusion is based on indirect inference only.

*The antihormone theory*. Collip (1934) put forward the theory that the antihormones represent physiological body constituents present, though in small quantities, in the normal blood and are necessary for the accurate regulation of hormone mechanisms in the normal organism. They help to regulate the action of

hormones and to counteract excessive stimulation due to a sudden discharge of large doses of a certain hormone into the blood stream. The main support of this theory was the observation that even the blood of non-pretreated human beings may contain antihormones.

*The site of antihormone formation.* It has been shown by numerous investigators that removal of the effector organ—in the case of the gonadotrophic hormone removal of the ovary or testis—does not significantly alter the ability of the organism to form antihormones (Bachman *et al.* 1934; Brandt & Goldhammer, 1936*a*; Sulman, 1937). Furthermore, even gonadotrophic preparations which have been inactivated by oxidation or ultraviolet light retain, as far as their action on the gonad is concerned, the ability of antihormone formation (Brandt & Goldhammer, 1938). It has been claimed, however, that splenectomy, especially when combined with blockade of the reticulo-endothelial tissue, inhibits antihormone formation (Gordon *et al.* 1937). (For a more detailed discussion of the evidence concerning this point, see section on "Stimuli influencing the antigonadotrophic hormone content of the blood".)

### (4) *Effect of antigonadotrophic hormone on various organs*

*The hypophysis.* It has been shown that chronic treatment with hypophyseal extracts causes the appearance of typical "signet-ring" castration cells in the pituitary of the rat, but only at a time when the ovaries have become entirely insensitive to the gonadotrophic extract and the ovarian atrophy has progressed so far that the theca cells are transformed into "wheel cells" (Collip *et al.* 1938).

*The gonads.* Although there is no indication of any direct effect of the antigonadotrophic hormones on the gonads, the early experiments of Selye *et al.* (1934*b*, *c*) and of Selye, Bachman, *et al.* (1934) have already shown that when the antigonadotrophic hormone titre of the blood reached a high level as a result of continued gonadotrophic hormone treatment, the gonads of the donor animals showed considerable atrophy. In certain cases, this atrophy progressed so far that wheel cells appeared in the theca (Collip *et al.* 1938), a finding which is characteristic of the complete absence of hypophyseal gonadotrophic stimuli as shown by Selye *et al.* (1933, 1934*a*). This is most probably due either to an inactivation of the gonadotrophic hormone secreted by the animal's own pituitary or to a complete inhibition of gonadotrophic hormone production. The same possibilities of interpretation should be considered in connexion with the demonstration by Parkes & Rowlands (1936) of the inhibition of ovulation following mating in rabbits treated with antigonadotrophic sera, and in connexion with the observation that these sera suppress spontaneous ovulation in the pubertal female and cause rapid atrophy of the reproductive organs in the male rat (Rowlands, 1937).

*The pregnant uterus.* According to Rowlands (1937) antigonadotrophic serum inhibits implantation of the blastocyst in the rabbit, or if administered later during gestation causes resorption of the foetuses. In the rat or mouse he observed no

such results. On the other hand, Bachman *et al.* (1934) found that rats rendered insensitive to the gonadotrophic hormone of pregnancy urine may still conceive, but in most cases pregnancy ended with death and resorption of the foetuses.

### (5) *The antigonadotrophic hormone content of the blood in normal animals*

In the *rabbit* Chen (1937) found that even the serum of normal non-pretreated animals may prove to be rich in antigonadotrophic hormone when tested on the immature female rat. In *man* numerous investigators were able to demonstrate the presence of antigonadotrophic hormones in the blood, both in the normal individual and in patients suffering from various diseases, but it has not as yet been possible to establish any clear correlation between the clinical condition and the antihormone content of the serum (Collip, 1935*a*; Collip & Anderson, 1935; Engel, 1935*a*, *e*; Laroche & Simonnet, 1936; Brandt & Goldhammer, 1936*a*; Twombly, 1936; Sulman, 1937).

### (6) *Stimuli influencing the antigonadotrophic hormone content of the blood*

*Gonadotrophic hormone treatment.* One of the most active means of increasing the antigonadotrophic titre of the blood is continued treatment with a gonadotrophic preparation.

In the *bird* Zavadovsky *et al.* (1937) noted testicular atrophy following a period of excessive development when young cocks received chronic treatment with a pregnant mare serum extract. This has been ascribed to antihormone formation.

In the *dog* an antigonadotrophic serum has been obtained by Thompson & Cushing (1937) by treatment with sheep pituitary extract for a period of about 4 months. Its potency was assayed in the rat.

In the *guinea-pig* Guyénot *et al.* (1937) found that treatment with cattle pituitary extracts led to the formation of antigonadotrophic hormone against the luteinizing factor, but not against the follicle-stimulating one. Treatment with a purely follicle-stimulating preparation made from the urine of castrate women led to no antihormone formation. They tested their sera on the guinea-pig against a gonadotrophic preparation of known potency.

In the *goat* Rowlands (1938*b*) obtained an antiserum against a human pregnancy urine preparation after 5 months of treatment. He assayed it on the oestrous rabbit.

In the *monkey* Meyer & Gustus (1935) and Gustus *et al.* (1935) demonstrated the presence of an antigonadotrophic effect beginning at the 27th day of treatment daily with 5 rat units of a gonadotrophic preparation of pregnant mare serum. They assayed the serum on immature rats. It is of interest that even serum obtained from a monkey 67 days after the injections of the hormone had been stopped, still proved to contain antigonadotrophic hormone. A refractory ovarian condition has also been noted in monkeys treated continuously with a sheep hypophysis extract, but, while this persisted in juvenile animals, it proved only temporary in adults.

In the *rabbit* Twombly & Ferguson (1934) obtained a potent antiserum against

gonadotrophic hormone, prepared from the urine of a patient suffering from teratoma testis, by injection with 100 mouse units daily over a period of $3\frac{1}{2}$ months. A similar antiserum against a pregnancy urine extract has been obtained by the injection of 75 mouse units daily for one month. The antisera were tested on the female mouse. Bachman *et al.* (1934) also obtained such an antiserum against pregnancy urine extract, the potency of which was tested on the immature rat. They emphasized that blood drawn 1–2 months after discontinuing treatment was no longer inhibitory. According to Brandt & Goldhammer (1936*a*) the blood of rabbits treated with a gonadotrophic preparation from human pregnancy urine begins to show antagonistic potency after about 3–4 weeks of treatment and loses it if treatment is discontinued for 6–8 weeks. Hamburger (1938) found that antigonadotrophic activity may be demonstrated beyond doubt in rabbit blood as soon as the 19th day of gonadotrophic hormone treatment.

The rabbit has been found to be a very satisfactory animal for the preparation of antigonadotrophic sera. They have been obtained in this species against ***human pregnancy blood*** extract, tested on the rat (Fluhmann, 1935*a*, *b*); human ***pregnancy urine extract***, tested on the rat (Engel, 1935*c*; Brandt & Goldhammer, 1936*a*; Zondek & Sulman, 1937*a*, *b*), the mouse (Kindermann & Eichbaum, 1936; Twombly, 1936) or the rabbit (Gegerson *et al.* 1936; Moricard, 1936; Rowlands, 1938*b*); against ***pregnant mare serum*** extract, tested on the rat (Zondek & Sulman, 1937*a*) and the rabbit (Rowlands, 1938*b*); against ***cattle pituitary*** extracts, tested on the rabbit (Gegerson *et al.* 1936; Parkes & Rowlands, 1936; Rowlands, 1937, 1938*b*); against ***horse pituitary*** extract, tested on the rabbit (Rowlands, 1938*b*); and, finally, against a ***relatively inactive human male urine*** extract, tested on the rat (Fremery & Scheygrond, 1937*a*).

The ***rat*** was the animal in which the existence of an antigonadotrophic hormone was discovered (Selye *et al.* 1934). The animals used were 21-day-old female rats injected daily with 100 rat units of a pregnancy urine extract over a period of 8 months. The antigonadotrophic effect of their blood was tested in the immature female rat. The subsequent work of Fluhmann (1935*a*, *b*) showed that similar antihormones may also be produced in the rat by pretreatment with human or sheep pituitary extract. These authors also used the immature rat as a test object.

In the ***sheep*** Collip (1937) was able to obtain a potent gonadotrophic antiserum by daily treatment with a sheep pituitary extract. The serum was tested against this same extract in the immature rat.

In ***man*** Sulman (1937) could find no antigonadotrophic hormone in the blood after parturition, that is, at a time when prolonged exposure to gonadotrophic hormones has occurred, nor could he demonstrate any such antihormone in the blood of a male patient who had undergone treatment with 12,000 rat units of pregnancy urine hormone within 9 months, or in a woman treated with 4200 rat units within 2 months. Similar findings have also been reported by Brandt & Goldhammer (1936*a*) and Spence *et al.* (1938). The latter found that even pig pituitary extracts failed to elicit antihormone formation in man. Dorff (1938) found, in a group of boys who had received urinary gonadotrophin for varying

degrees of genital underdevelopment, and treated for varying periods of time (2–16 months), no antihormone to be present.

*Ovariectomy.* Removal of the gonads does not prevent the formation of antigonadotrophic substances in the female rabbit (Bachman *et al.* 1934; Brandt & Goldhammer, 1936*a*; Sulman, 1937) and castration in the male is likewise without effect on the development of these antihormones (Sulman, 1937).

*Splenectomy.* Removal of the spleen, especially when combined with blockage of the reticulo-endothelial system, inhibited the formation of antihormones against a gonadotrophic preparation of human pregnancy urine, or sheep pituitary, according to Gordon *et al.* (1937, 1938). They concluded that the reticulo-endothelial system is probably the site of antihormone formation.

*Pregnancy.* According to Cole & Hart (1930) the blood of pregnant mares contains a principle which inhibits ovarian development when injected into immature rats. This inhibitory substance, which appears after the 150th day of gestation, is not identical with oestrin, but its nature has not yet been definitely established.

### (7) *The antigonadotrophic hormone content of tissues other than blood*

*The hypophysis.* Reiss *et al.* (1931*a*, *b*, *c*) showed that the gonadotrophic effect of pregnancy urine extracts may be inhibited by relatively purified preparations of the anterior lobe which had been freed of growth hormone. They were able to show this antigonadotrophic effect in both immature male and female rats. Evans *et al.* (1936) described a method for the further purification of this principle, which they call the pituitary "antagonist". It remains to be shown whether the principle in question is identical with the antigonadotrophic hormone which appears in the blood of animals treated with gonadotrophic preparations.

*The pineal gland.* Engel (1934, 1935*a*, *b*, *c*, *d*, *e*) and Engel & Buno (1936) claimed that extracts of the pineal gland of various animals contain a substance which is an extremely active antagonist of the gonadotrophic hormone, and they expressed the opinion that the pineal gland may possibly be the organ which produced the antigonadotrophic hormone. Zondek & Sulman (1937*a*) were unable, however, to confirm these findings.

*Urine.* According to Durupt *et al.* (1935) antigonadotrophic hormone may appear in the urine and they showed in a woman suffering from dysmenorrhoea that relatively large amounts of such an antagonist are excreted since extracts of her urine were active in preventing the gonadotrophic effect of human pregnancy urine in the immature mouse. Brandt & Goldhammer (1936*a*), on the other hand, were never able to demonstrate antigonadotrophic hormone in the urine of their patients.

## III. THE PROGONADOTROPHIC HORMONE

Although it seems rather questionable whether this subject should be discussed here, we shall review the literature on the so-called progonadotrophic principle together with the antihormones, because of the fundamental similarities which appear to exist between them.

It was shown by Collip (1937) that the serum of sheep injected for short periods with sheep pituitary extract may augment the action of the injected extract in test rats, and similar observations have been made by Thompson (1937), who obtained such augmentary sera in the horse and dog following treatment with sheep pituitary gland. Thompson considered this effect to be the result of the formation of an antihormone to the pituitary antagonist. Rowlands (1938*a*, *b*) confirmed these experiments in sheep receiving sheep pituitary extracts, and in a goat treated with a pig pituitary extract. He is inclined to agree with Thompson's interpretation of this observation. Guyénot *et al.* (1937) claimed that the serum of guinea-pigs chronically injected with cattle pituitary extracts, though inhibitory to the luteinizing principle, caused follicle maturation when injected by itself. It is difficult to decide whether this effect was due to an augmentation of the effect of the animal's own pituitary or simply to the presence of a residue of the injected follicle-stimulating principle. Katzman *et al.* (1937) were apparently the first to note progonadotrophic effects.

## IV. THE ANTITHYROTROPHIC HORMONE

### (1) *Chemistry*

Collip & Anderson (1935) state that boiling at *p*H 5 for 3 min. completely destroys the antithyrotrophic activity of serum, and a considerable loss of activity occurs even if the serum is simply preserved in sterile ampules in the refrigerator for 2 months. They (Anderson & Collip, 1934) claimed to have obtained antithyrotrophic extracts by precipitating the serum proteins with 50% acetone. Fellinger (1936) mixed blood with gypsum and then dried it so that it could easily be crushed to make a fine powder, which was then extracted with ether. Such an ether extract of 100–120 c.c. of normal blood inhibited the action of $2\frac{1}{2}$ units of thyrotrophic hormone in the guinea-pig. It is most unlikely that this lipid extract is identical with the antithyrotrophic hormone which appears in the serum of thyrotrophic hormone-treated animals. Harington & Rowlands (1937) claimed that the antithyrotrophic hormone, which appears in the serum of rabbits chronically injected with an ox anterior pituitary extract, is non-dialysable and almost completely precipitated by $\frac{1}{3}$–$\frac{1}{2}$ saturation with ammonium sulphate, but not by dilution and adjustment to *p*H 5·5. The activity, therefore, appears to be associated with the pseudoglobulin fraction. These authors showed also that the dried pseudoglobulin fraction retains its full activity when kept as a powder for over a year. Loeser & Trikojus (1937) state that the antithyrotrophic principle may either be adsorbed to animal charcoal or to the precipitate obtained by introducing an alcoholic solution of benzoic acid into blood serum, and that the active substance may readily be eluted from these adsorbates.

### (2) *Bio-assay*

In the *rat* and *guinea-pig* Collip & Anderson (1934) availed themselves of the inhibition of the *metabolic response* to thyrotrophic hormone as an indicator of the antithyrotrophic potency of inhibitory serum.

Loeser (1936*a*) and Eitel & Loeser (1934, 1935*a*) injected antithyrotrophic serum intraperitoneally over a period of 6 days and, together with the last two of these injections, they administered a known amount of thyrotrophic hormone. The prevention of *histological signs of thyroid stimulation* is their criterion of antithyrotrophic activity. A similar test is used by Oudet (1936*a*), who draws blood from the thyrotrophic hormone resistant donor 4 days after the last hormone injection (so as to make sure that all the injected thyrotrophic hormone has disappeared) and then injects this serum into immature guinea-pigs. Two days later, the guinea-pigs are treated with a known amount of thyrotrophic hormone simultaneously with non-pretreated controls. After 2 more days, all animals are sacrificed and their thyroids examined histologically.

Harington & Rowlands (1937) injected the antithyrotrophic serum simultaneously with the thyrotrophic preparation once daily to five immature guinea-pigs of 200 g. body weight during 5 days, at the end of which time the *increase in thyroid weight* was used as an indication of active stimulation.

In the *rat* Collip & Anderson (1935) found that the inhibition of the metabolic response to thyrotrophic hormone is a good criterion of antithyrotrophic activity. Both normal and hypophysectomized rats may be used for such assays.

### (3) *Theories*

#### (*a*) *Specificity of the antithyrotrophic hormones.*

The question of *species specificity* has not yet been thoroughly investigated in the case of the antithyrotrophic hormone, though Eichbaum & Kindermann (1936) found that rabbits or guinea-pigs became resistant to cattle or pig pituitary thyrotrophic extracts. This resistance extended only to the extract with which the animals were pretreated. This has been confirmed by Eichbaum *et al.* (1937) and Oudet (1937*a*). It has been claimed, however, that after very long periods of pretreatment this apparent species specificity vanishes again (Oudet, 1937*b*). More recently it has been claimed that rabbit serum extract renders guinea-pigs resistant to the action of cattle pituitary thyrotrophic preparations (Oudet, 1938). There can be no question of *organ specificity* in this case since potent thyrotrophic preparations can only be obtained from hypophyseal tissue. There is, however, some evidence of *extract specificity*, for Werner (1936*a*, *b*) has shown that if two thyrotrophic extracts were prepared from cattle pituitary tissue, one by means of the sodium sulphate, the other by the flavianate method, and guinea-pigs were injected with the same number of thyrotrophic hormone units of each of these preparations, the thyroids of animals which had become refractory to the sodium sulphate preparation were still sensitive to the flavianate preparation, while the flavianate preparation itself had little if any immunizing effect when given in equivalent doses. The question of *hormone specificity* is not sufficiently clarified as yet. While Anderson & Collip (1934) showed that antithyrotrophic sera do not influence the metabolic action of thyroxine, it appears that "tyronorman", an extract of normal blood, inhibits the actions both of the thyroid and the thyrotrophic hormones (Romeis,

1923; Herzfeld & Frieder, 1933; Schöneberg, 1933; Baumann, 1934; Schneider & Widmann, 1935; Blum, 1932).

(*b*) *Nature of the antithyrotrophic hormone.*

The above-mentioned experiments of Werner (1936*a*, *b*), who found that of two preparations of ox pituitary thyrotrophic hormone made by different methods, one may be very active in causing antihormone formation while the other has almost no such effect although its potency is the same, have been interpreted as a strong argument in favour of the conception that antihormones are antibodies formed against the partially denatured protein of the hormone extract. Max *et al.* (1935) also concluded that the antithyrotrophic hormone probably is an antibody, although they do not state quite clearly what experimental evidence led them to this conclusion. Eichbaum & Kindermann (1936) concluded that the antithyrotrophic hormone is probably an antibody because it is species-specific and there is a close parallelism between the antibody concentration in the blood and its antihormone activity. The detailed serological analysis of the antibodies which appear in the blood of thyrotrophic treated animals showed species-specific antibodies, which react with the serum protein of the species with whose pituitary the animals were pretreated, and apparently hormone-specific antibodies which react with thyrotrophic preparations of various animals (Eichbaum *et al.* 1937).

Contrary to these investigators, Collip (1934) hesitates to regard the antithyrotrophic hormone as a true serological antibody. Similarly, Scowen & Spence (1934, 1936) claimed that it is inconceivable that an animal should form an antibody against its own hormone and they state that the presence of antithyrotrophic hormone in the blood of normal rabbits and men is a certain proof against the antibody theory. The detection of antithyrotrophic hormone in the normal blood of various species also led Eitel & Loeser (1935*a*, *b*) and Loeser (1936*a*, *b*) to reject the antibody theory and to regard the antithyrotrophic hormone as a physiological body constituent.

It should be mentioned in this connexion that according to Loeser (1936*c*) and Eitel & Loeser (1935*b*) thyroidectomy prevents antithyrotrophic hormone formation while thyroxine restores it again, so that these authors are inclined to regard the antithyrotrophic principle as identical with the thyroid hormone. Gessler (1937), on the other hand, was able to prevent both the metabolism-stimulating and the thyrotrophic effect of pituitary preparations in hypophysectomized rats by simultaneous treatment with oestrin.

If the findings of Shkhvazabaia (1938) receive confirmation they might modify our conception of the chemical nature of antithyrotrophic substances, since this author claimed that adrenaline and numerous alkaloids (atropine, cocaine, morphia, nicotine, eserine, ergotamine) are capable of preventing the thyroid-stimulating effect of hypophyseal extracts in the guinea-pig, and similar results have been obtained with barbiturates by Voges (1936). While ascorbic acid may neutralize the effect of thyroid hormone, it has no antithyrotrophic effect (Eitel, 1938*b*) and consequently cannot be held responsible for the antithyrotrophic activity of the blood.

### (4) *Effect of the antithyrotrophic hormone on metabolism and on various organs*

*Metabolism.* The basal metabolism of normal rats decreases below the normal level under the influence of antithyrotrophic hormone injections and the metabolic effect of thyrotrophic hormone is inhibited (Anderson & Collip, 1934). "Tyronorman", on the other hand, exerts no definite action on the basal metabolism of normal animals although it inhibits the rise otherwise obtained by thyrotrophic extracts (Keil, 1936; Zimmermann, 1936).

*The glycogen content of the liver* is not significantly influenced by tyronorman in normal animals (Zimmermann, 1936; Loeser, 1936*a*), but the depletion of liver glycogen otherwise caused by thyrotrophic hormone is prevented (Schneider & Widmann, 1935). It is of interest that the liver of guinea-pigs chronically treated with thyrotrophic hormone regains its glycogen content—which vanished during the first few days of treatment—much sooner than the thyroid loses its responsiveness, as judged by its histological appearance (Fischbach & Terbrüggen, 1938).

*Thyroid.* Eitel & Loeser (1935*a*) showed that antithyrotrophic sheep serum causes atrophy of the normal thyroid in the guinea-pig, and it is of course well known that the thyroid-stimulating effect of thyrotrophic extracts is inhibited by it (Schneider & Widmann, 1935; Loeser, 1936*c*; Oudet, 1936*a*).

*Hypophysis.* The thyrotrophic hormone content of the hypophysis decreases under the influence of treatment with antithyrotrophic sera (Anderson & Collip, 1934; Eitel & Loeser, 1935*a*).

### (5) *The antithyrotrophic hormone content of the blood in various species*

Loeser (1936*b*) showed that the serum of normal *dogs* contains a certain amount of antithyrotrophic activity. Schneider & Widmann (1935), Loeser (1936*b*), and Glaubach & Pick (1937) found a high degree of antithyrotrophic potency in "Tyronorman". As far as we are aware, none of the publications reveal, however, the species from which the blood is obtained for the preparation of this extract. Eitel & Loeser (1935*a*) also showed antithyrotrophic activity in normal *sheep* serum. The fact that normal *rabbit* serum (Scowen & Spence, 1936) and normal *human* serum (Herold, 1934*a*, *b*; Collip & Anderson, 1935; Fellinger, 1936; Eitel, 1938*a*; Scowen & Spence, 1936) may likewise contain antithyrotrophic activity has also been established, though Rathcke (1938) was unable to demonstrate it in the rabbit.

### (6) *Stimuli influencing the antithyrotrophic hormone content of the blood*

*Thyrotrophic hormone treatment.* In the *English sparrow* even prolonged thyrotrophic hormone treatment will not lead to refractoriness or antihormone formation, according to Miller (1938).

In the *dog* Collip & Anderson (1935) obtained an antithyrotrophic hormone, the potency of which was assayed in the guinea-pig. These findings have been confirmed by Loeser (1936*b*, *c*).

In the *horse* chronic treatment with thyrotrophic hormone has likewise been shown to result in the development of antithyrotrophic activity in the serum (Collip & Anderson, 1935; Anderson & Collip, 1934).

In the *guinea-pig* antithyrotrophic sera have also been obtained by several investigators following chronic administration of the thyrotrophic hormone. In all these cases the serum was assayed in the guinea-pig (Collip & Anderson, 1935; Eichbaum & Kindermann, 1936; Oudet, 1936*a*, *b*).

In the *rabbit* the appearance of antithyrotrophic substances following treatment with thyrotrophic extracts of various species is also well established, the thyrotrophic hormone neutralizing power of the sera when transferred to guinea-pigs being the most commonly used test object (Collip & Anderson, 1935; Eichbaum & Kindermann, 1936; Rowlands & Parkes, 1936; Harington & Rowlands, 1937; Eichbaum *et al.* 1937; Scowen & Spence, 1934, 1936; Oudet, 1936*a*, *b*, 1937*a*, *b*; Pighini, 1937; Chou, 1937). Rowlands & Parkes (1936) tested the antithyrotrophic activity at various intervals during treatment with the thyrotrophic hormone, and found that activity appears in the rabbit about the fourth week and reaches a maximum at the tenth week, when 2 c.c. of the rabbit serum inactivates an amount of thyrotrophic extract which would otherwise suffice to double the weight of the thyroid of immature guinea-pigs. According to Okkels (1937) the serum of rabbits rendered resistant to thyrotrophic hormone antagonizes the stimulating effect of this hormone on thyroids perfused by the Lindbergh pump.

In the *rat* Collip & Anderson (1935) demonstrated the appearance of antithyrotrophic hormone in the blood, showing that this serum given in quantities of 0·5–1 c.c. twice daily for 3 days to hypophysectomized rats prevented a rise in metabolic rate with amounts of thyrotrophic hormone equal to 200 times the minimum effective dose. The potency of this serum has also been shown using normal rats or guinea-pigs as test objects (Collip & Anderson, 1934).

In the *sheep* Eitel & Loeser (1935*a*) made the interesting observation that in the case of continuous treatment with thyrotrophic hormone, the antithyrotrophic titre of the blood reached a maximum between the fourth and fifth week, after which a gradual decline ensued, and at the end of 9–12 weeks the antithyrotrophic effect of the serum vanished in spite of continued treatment.

In *man*, given therapeutic injections of thyrotrophic hormone, Collip & Anderson (1935) were also able to demonstrate the presence of antithyrotrophic activity in the blood.

*Hypophysectomy.* The removal of the hypophysis does not interfere with the development of resistance against the thyrotrophic hormone (Gessler, 1937) and the appearance of antithyrotrophic substances in the blood of the rat (Collip & Anderson, 1935) or dog (Loeser, 1936*b*, *c*).

*Thymectomy* increases the antithyrotrophic titre of rabbit blood, according to Rathcke (1938).

*Thyroidectomy.* Removal of the thyroid decreases the ability to form antithyrotrophic hormone in the sheep, and if the thyroid is removed at a time when the antithyrotrophic titre of the serum has already been raised by thyrotrophic hormone

treatment a sudden decrease in antithyrotrophic activity results. Treatment with thyroxine restores the antihormone forming ability of the blood, according to Loeser (1936*c*) and Eitel & Loeser (1935*a*, *b*) who concluded that the development of resistance against the thyrotrophic hormone is largely dependent upon the thyroid. These findings might explain the observation of Aron (1930), who showed that thyroid hormone inhibits the thyroid-stimulating effect of thyrotrophic hormone in the guinea-pig. However, Oudet (1936*b*), apparently unaware of the previous findings of Eitel & Loeser, claimed that thyroidectomy does not significantly influence the formation of antithyrotrophic hormone in guinea-pigs or rabbits treated with the thyrotrophic principle, and this was confirmed for the rabbit by Chou (1937). In *Graves's disease* and *during gestation* the antithyrotrophic activity of human blood is subnormal (Herold, 1934). This has been confirmed for hyperthyroidism by Eitel (1938*a*), while in *carcinoma* patients the latter investigator found unusually high concentrations of antithyrotrophic substance in the blood.

### (7) *The antithyrotrophic hormone content of tissues other than the blood*

Collip & Anderson (1934) showed that saline extracts of the liver and spleen of thyrotrophic hormone-resistant rats may also prevent the rise in metabolism normally caused by thyrotrophic hormone injections.

## V. THE ANTITHYROID HORMONE

### (1) *Chemical nature and theories*

The consensus of opinions concerning the chemical nature of the antithyroid principle contained in normal blood is that the substance belongs to the *lipids* and consequently may be extracted from the blood by lipid solvents (Romeis, 1923; Blum, 1932; Saegesser, 1933; Schneider & Widmann, 1935). According to Schneider & Widmann (1935) this lipid-soluble material present in normal blood is identical with both the antithyroid and the antithyrotrophic hormone, since besides antagonizing the effects of thyroxin it also neutralizes the thyroid-stimulating effect of the thyrotrophic hormone. It should be emphasized, however, that the antithyrotrophic principle of Collip & Anderson is protein-like in nature, inactive by mouth, and unable to inhibit the action of thyroxine. It should therefore not be identified with the lipid-soluble, orally active principle contained in normal blood.

The active principle of Antithyroidin-Möbius, prepared from the blood of thyroidectomized sheep, is in the euglobulin fraction, according to Gürber & Geszner (1928). A method for the preparation of antithyroid principle from the blood of similarly treated goats has been described by Asimoff (1926*a*).

According to Kraft (1936) 3-*fluortyrosine* in doses of 700$\gamma$ (containing 70$\gamma$ of fluorine) inhibits the action of 15$\gamma$ of thyroxine in the tadpole metamorphosis test. Since this author found about 15–100$\gamma$ fluorine in 100 c.c. of normal human blood, he thinks that this element, possibly in the form of a compound such as fluortyrosine, may be the physiological antagonist of thyroxine which occurs in normal blood. He emphasizes that tyronorman and a similar blood extract called "solvitren" are

rich in fluorine. Litzka (1936*a*, *b*) showed that while fluorine and tyrosine have only a slight thyroid hormone antagonizing effect, fluortyrosine is several hundred times more active in this respect than either of its constituents and counteracts all the important physiological effects of thyroxine in various biological tests. It has no antithyrotrophic effect, however.

According to Kin (1938) pancreatectomy prevents the formation of antithyroid substance in the thyroid hormone-treated rabbit. He concludes that the thyroid hormone neutralizing principle is a hormone which originates in the pancreas.

It is not within the scope of this review to give a detailed discussion of all the substances which have been found to antagonize the action of thyroid hormone, but we wish to emphasize that ascorbic acid, diiodotyrosine, various sterols, and numerous other substances have been found to possess such an activity.

The question whether a true immunity with *antibody* formation may occur as a defence against thyroid extracts was first investigated by Papazolu (1911). This author obtained a positive complement fixation reaction with the serum of patients suffering from Graves's disease against extracts of the thyroids of such patients. Vallagussa (1910) and Kolle (1909) were unable, however, to demonstrate any antibody or antigen in the blood or thyroid of patients suffering from Graves's disease. Ballner *et al.* (1912) found that sera of individuals suffering from goitre, and even sera of non-goitrous individuals, living in the Tyrol where goitre is endemic, give a complement fixation reaction with alcoholic extracts of thyroid tissue. Bauer *et al.* (1937), Bauer (1937), Bauer & Kunewälder (1937) and Bauer & Schächter (1936) found that rabbits chronically treated with thyroxine become resistant to the hormone, and at the same time their serum acquires the property of giving a positive complement fixation reaction with thyroxine or diiodotyrosine. However, they found that this resistance cannot be transferred passively by injecting the serum of a thyroxine-resistant animal into a non-pretreated one. These authors observed, furthermore, that the complement fixation reaction with diiodotyrosine, which is usually positive in cases of severe Graves's disease, became negative following the removal of the hyperactive thyroid. Kestner (1937) reported experiments showing that the serum of sheep chronically injected with pig thyroid emulsions exerts antithyroid effects, in so far as rabbits, dogs and rats treated with this serum show a decrease in metabolic rate. The author believes this to be due to the formation of organ-specific antibodies.

The positive results of immunizing experiments against thyroglobulin (Hektoen *et al.* 1927; and others) have already been mentioned in the historical introduction and need not be discussed here in detail, since there is little evidence of their having anything to do with antihormones in the true sense of the word, especially since it was shown that *in vivo* antithyroglobulin fails to antagonize the physiological actions of thyroglobulin (Schulhof, 1930). Rosen & Marine (1937) showed that prolonged injections of iodothyroglobulin do not cause refractoriness to the metabolic action of this substance in the rabbit, in spite of the definite immune response (precipitin formation). It also suffices simply to mention the finding of Picado & Rotter (1936), who obtained specific precipitins against thyroid protein.

### (2) *Effect of the antithyroid principle on metabolism and on various organs*

It is obviously impossible to discuss the physiological effect of the antithyroid hormone at this stage when even its very existence has not as yet been definitely proven. It might be useful in this connexion, however, to enumerate the most important effects of thyroid-hormone-antagonizing blood extracts without taking any definite stand for or against their interpretation as true antihormones.

The fact that tyronorman decreases the rise in *basal metabolism* normally caused by the thyroid hormone seems to have been well established both in animal experiments and in patients suffering from Graves's disease (Blum, 1932, 1933; Schöneberg, 1933; Herzfeld & Frieder, 1933; Mayer-Umhöfer, 1932; and Baumann, 1934). According to Oberdisse & Thaddea (1932*a*, *b*) even the metabolism of normal, otherwise non-treated rats decreases 16–26% below normal under the influence of treatment with Antithyroidin-Möbius.

The *glycogen fixing ability of the liver* is not significantly influenced by tyronorman in the normal rat or guinea-pig (Zimmermann, 1936). On the other hand, Anselmino & Hoffmann (1933) claimed that the depletion of liver glycogen, which is normally caused by thyroxine treatment in the rat, may be prevented by tyronorman feeding, and similar results have been obtained in the guinea-pig by Schneider & Widmann (1935). Loeser (1936*a*), however, was unable to show such an antagonism between thyroxine and tyronorman with respect to their action on the liver glycogen of the guinea-pig.

The *metamorphosis*-stimulating effect of thyroxine may also be inhibited by blood or lipid extracts of blood (Romeis, 1916; Blum, 1932; Eufinger & Gottlieb, 1935; Eisfeld, 1936; etc.).

Küstner (1934) claimed that tyronorman increases *milk secretion* in women, a fact which he explained as resulting from a neutralization of excess thyroid hormone, which, as is known, may decrease milk production. Hinrichs (1935) was unable, however, to confirm these findings.

### (3) *The antithyroid hormone content of the blood and tissues under normal conditions and under the influence of various stimuli*

The ether-soluble fraction of normal human urine contains an antithyroid principle, according to Keeser (1938).

Fellinger & Schlesinger (1935) showed that the ether-soluble antithyroid substances of the blood disappear under the influence of Graves's disease, and reappear if such patients are successfully treated by X-ray irradiation of the thyroid or by thyroid resection. This increase to normal or even above normal level was particularly marked in cases treated with diiodotyrosine. In normal persons, on the other hand, Bodart & Fellinger (1936) found no increase in the antithyroid substance content of the blood following diiodotyrosine treatment. The latter investigators found, furthermore, that in myxoedema, the blood is particularly rich

in such antithyroid substances and thyroid treatment decreases their concentration in such cases.

Magistris (1935) claimed to have isolated a water-soluble antithyroid compound from the blood of rats rendered resistant to thyroid hormone by continued thyroid treatment. He claims that this principle is identical with the metabolic hormone of the anterior lobe which he calls "orophysin" and differs from the "catechin" of Blum. He observed, furthermore, that treatment with anterior lobe extracts containing orophysin antagonizes the action of thyroid hormone on the liver glycogen of the rat. This observation confirmed him in his belief that the hypophysis contains and produces an antithyroid principle.

According to Kin (1938) *pancreatectomy* prevents the formation of antithyroid substances in the blood of the rabbit.

## VI. ANTIHORMONES AGAINST VARIOUS OTHER HORMONES

### (1) *Antigrowth hormone*

Collip (1934) noted that hypophysectomized rats treated with a purified growth hormone preparation grew rapidly for a certain time but then their growth ceased in spite of continued treatment with the same dose of the same extract. Evans *et al.* (1935) confirmed these experiments with purified preparations, but found that no such decline in growth occurred in hypophysectomized rats treated with crude extracts of growth hormone. Since they observed that such crude extracts re-initiate growth in hypophysectomized animals after they ceased to grow under the influence of treatment with purified growth hormone preparations, they concluded that the cessation of growth in rats treated with purified preparations is not due to the development of antihormones, but to the necessity of substances, which are present in crude extracts but not in the purified ones, for continued growth.

### (2) *Antiprolactin*

Following continued treatment with a prolactin preparation pigeons may become insensitive to large doses of this particular extract. Since, however, they will still respond to relatively small doses of other prolactin preparations, the phenomenon was considered an extract-specific immunity rather than a loss of sensitivity to prolactin itself (Kabak & Stulova, 1937). In goats the milk production-stimulating effect of anterior lobe extracts likewise decreases after some time (Grüter, 1931). Following prolonged treatment of rabbits and monkeys with a prolactin preparation which also contained the "glycotrophic factor", Young (1938) obtained an anti-serum which antagonized the effect of prolactin on the crop gland of the pigeon. The serum did not inhibit the glycotrophic effect of pituitary extract, but it had a marked antithyrotrophic effect, although the antigenic pituitary extract contained no demonstrable amounts of thyrotrophic hormone. Strangeways (1938) noted that an antiprolactin serum obtained from rabbits precipitates prolactin solutions *in vitro* and at the same time decreases their activity on the crop gland.

### (3) *The antiketogenic hormone*

Black *et al.* (1934) showed that rats treated chronically with hypophyseal extracts rich in the ketogenic principle become very resistant to these extracts, and their serum, when injected into otherwise untreated animals, transfers to them the resistance against the ketogenic action of pituitary extracts. It was shown, furthermore (Black, 1935), that rats resistant to the ketogenic principle showed only slight acetonuria when treated with large doses of phlorrhizin while fasting. Black interpreted his findings as showing that phlorrhizin causes ketonuria by stimulating the hypophysis to secrete more ketogenic hormone, and that pretreatment with ketogenic extracts prevents this ketonuria, because it renders the animals resistant to the ketogenic hormone secreted (under the influence of phlorrhizin) by their own pituitaries. If this interpretation is correct, his findings would give very strong support for the antihormone theory.

### (4) *Anticortin*

Hartman *et al.* (1938, 1939) showed that constant treatment with cortin leads to a refractory condition in dogs and man which may be transmitted from individual to individual by blood transfer. The immune serum gives positive complement fixation and precipitation reactions with the antigenic cortin preparation (Toby & Lewis, 1937).

### (5) *Antioestrin*

D'Amour *et al.* (1934) and Twombly (1936) called attention to the fact that even prolonged treatment of castrate females with oestrin did not result in any decrease in their sensitivity to this hormone, and that the blood of such animals when transferred to other animals has no oestrin-antagonizing effect. They concluded that no antihormones are formed against non-protein hormones, which might be considered as an argument in favour of the antibody theory of antihormones. Ssargin & Pleteneva (1937) likewise failed to demonstrate antioestrin in the blood of rabbits chronically treated with large doses of oestrin. It should be emphasized, however, that in the non-castrate female there is a definite reaction to large doses of oestrin which consists of an enlargement of the adrenals, corpora lutea and the hypophysis (Selye *et al.* 1935). It has also been shown that this reaction is of great physiological significance, since adrenalectomized or hypophysectomized rats are extremely sensitive to oestrin and succumb after a relatively short treatment with this hormone (Selye *et al.* 1936). While it is impossible to say as yet whether the compensatory secretion of true hormones, in an endeavour to antagonize the action of excessive quantities of other hormones, should in any way be compared with antihormone formation, it appears from these experiments that oestrin may stimulate the production of certain oestrin-antagonistic substances. Selye *et al.* (1935) have also shown that after a prolonged period of oestrin treatment the enlarged corpora lutea involute, and, at least to a certain extent, the hypertrophy of the adrenals and the hypophysis retrogresses in spite of continued oestrin

administration. This finding may either be due to a loss of the oestrin-sensitivity of the hypophysis, or to the formation of anti-substances against the hormones secreted by the animal's own pituitary under the influence of oestrin. They cannot be regarded, however, as due to the formation of antihormones against oestrin itself, since the uterine and vaginal effect of the hormone persisted throughout the experiment.

It is interesting in this connexion that Brandt & Goldhammer (1936*b*) were able to produce complement-fixing antibodies against oestrin by combination immunization, using pig serum as a carrier. These antisera failed, however, to neutralize the physiological actions of oestrin.

### (6) *Antiprogesterone*

It is well known that progesterone is not able to maintain the endometrium in a decidual condition indefinitely, but this phenomenon of desensitization is not due to antihormone formation since the blood of rabbits which had become insensitive to the hormone of the corpus luteum as a result of chronic treatment fails to neutralize the action of progesterone in non-pretreated animals (Ostergaard, 1937*a*). In combination with oestrin, progesterone retains its activity for a very long time (Ostergaard, 1937*b*).

### (7) *Antitestis hormone*

Picado (1936) and Picado & Rotter (1936) claim to have demonstrated precipitins against testis protein in the blood of various animals, and speculate concerning their relationship to antihormones and to longevity. Brandt & Goldhammer (1936*b*), using pig serum as a carrier, produced complement-fixing antibodies against testis hormone (Proviron) which, however, did not neutralize the actions of the hormone in bio-assays.

### (8) *Other antihormone-like substances of doubtful nature*

The initial hyperglycaemia elicited by certain insulin preparations has been regarded as due to the presence of *anti-insulin* in the pancreas (Wichels & Lauber, 1932). Parturier (1935) claims to have produced an antiserum against *parathyroid hormone* which has beneficial effects in cases of asthma, rheumatism, etc., but his evidence is not very convincing. Kestner (1938) claims that the serum of rabbits chronically injected with a cattle pituitary emulsion causes a decrease in the specific dynamic action of the anterior lobe in rabbits. He attributes this to the formation of *organ-specific antibodies against anterior lobe tissue*. Following prolonged treatment with pituitary extracts containing the carbohydrate metabolism-influencing principle, rats become entirely resistant to its action. The cause of this resistance has not been analysed as yet (Russell, 1938). Serio (1936) found that dogs treated with *adrenaline* or *pituitrin* may go into chronic hypoglycaemia following a short period of glycosuria. The author debates the possibility that this change may be due to the formation of antihormone against these hormone preparations. A certain

refractoriness to adrenaline following prolonged pretreatment had already been observed by Elliott & Durham (1906) who emphasized, however, that this is not due to the formation of an antibody against the hormone. Ssaweljew (1904) claimed, however, that passive immunization against adrenaline is possible and Stradiotte (1905–6) asserts that he obtained an adrenaline-precipitating and neutralizing antiserum. Bauer (1937) obtained a positive complement-deviation reaction following chronic treatment with adrenaline, and similar results have been reported by Taubenhaus & Medak (1937), who used other phenol derivatives. A gonad-inhibitory *ovarian extract* was prepared by Schirch (1938), but the author does not think that this substance should be called an antihormone.

Numerous other examples of acquired resistance against various hormone preparations could be enumerated, among which the so-called "tachyphylaxis" against posterior lobe preparations is particularly well known, but since there is no definite proof of any direct connexion between these phenomena and antihormone formation, it seems hardly justified to give them space here. The same is true of the so-called cytotoxic sera and protective ferments.

## VII. SUMMARY

After a short review of the history of antihormones, the antigonadotrophic and antithyrotrophic hormones have been discussed in detail because these are the best known members of this group. The possible existence of other antihormones, especially of antigrowth hormone, antiprolactin, antiketogenic, anticortin, antithyroid hormone, etc. has also been considered.

In view of the fact that many of the relevant publications have appeared in foreign languages and in journals not readily accessible to the general public, we tried to make this review as complete as possible. We felt that in the present state of our knowledge, an unbiased presentation of all the available data which may throw light on the theoretical interpretation and practical evaluation of the antihormones would prove of more value to the reader than an elaborate discussion of theories and personal views, most of which would after all have to be based on impressions rather than on knowledge.

The most important facts which, as our survey shows, are not definitely established are the following:

1. The blood of animals chronically treated with gonadotrophic or thyrotrophic preparations acquires the ability to neutralize the action of such preparations in other animals.

2. The antihormones are specific in so far as, for instance, antigonadotrophic hormone does not antagonize the action of the thyrotrophic hormone and vice versa.

3. The blood of an animal rendered immune to a certain type of a gonadotrophic preparation, though very active in neutralizing the action of this preparation, may be relatively inactive with regard to other gonadotrophic extracts. Similar examples of extract specificity have been observed concerning the antithyrotrophic hormone.

4. Neither the presence of the effector organ (the gonads in the case of the gonadotrophic and the thyroid in the case of the thyrotrophic hormone) nor that of the hypophysis is essential for antihormone formation.

5. There is little or no evidence that chemically pure hormone preparations such as the oestrogens, adrenaline, etc., may form antihormones in the true sense of the word, although the sensitivity of the organism to such preparations may decrease after prolonged treatment.

In summarizing the essence of this survey, the authors emphasize, however, that the fact that *pretreatment with certain hormones will cause the appearance in the blood of principles antagonistic to these hormones* may be regarded as established beyond doubt. It is felt that the much-debated question, whether these principles are hormones or antibodies, cannot be answered with certainty as yet; and even if we knew more about them the answer would largely depend on our definition of these two types of active principles.

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# THE GENETICS AND CHEMISTRY OF FLOWER COLOUR VARIATION

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(*Received* 16 *March* 1939)

CONTENTS



## I. THE CHEMISTRY OF FLOWER COLOUR VARIATION

Colour is one of the characters of plants and animals most frequently used in genetical investigations. Separation of colour types depended until recently on visual comparisons alone. These, since they represent only a first analysis, are always inadequate and may sometimes be misleading. For a further understanding of the developmental processes involved we need a knowledge of the chemical structure and properties of the pigments responsible.

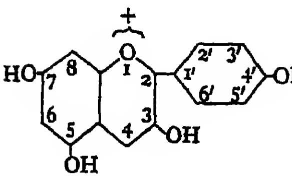
It is with the flower pigments, especially the anthocyanins and anthoxanthins, that chemical analysis has gone farthest. In other cases, such as eye colours in *Drosophila* (Beadle & Ephrussi, 1936, 1937) and chlorophyll deficiencies in *Zea Mays* (Phipps, 1929), *Trifolium pratense* (Williams, 1937) and *Datura* (Inman & Blakeslee, 1938), something is known of the pigments which are affected, but nothing of the nature of the changes they undergo. It is in the flower pigments that gene action can be examined for the first time in its fundamental sense, namely as governing simple chemical changes: oxidation, reduction, methylation and glycoside formation.

The great majority of flower pigments belong to three main classes, the anthocyanins and the anthoxanthins, both of which are sap-soluble, and the carotinoids, which are generally found in the plastids and are not sap-soluble. The term anthocyanin, introduced by Marquart (1835) and used by other early workers, denoted simply the red and blue sap-soluble pigments of plants. It is still used in this broad sense by botanists, but in chemical usage the term is generally reserved

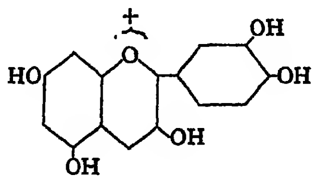
for what are known as the hydroxy-flavylium salts, which include most, but not all, of the red and blue plant pigments. Full details of the chemistry of the hydroxy-flavylium salts can be found in the publications of Willstätter (1913, 1914, 1915, 1916), Robinson[1] and Karrer[2] and their collaborators; as much as is relevant to this review is outlined below.

### (1) *The anthocyanins*

With two exceptions the anthocyanins (in the limited sense) are derived from three hydroxy-flavylium salts, pelargonidin (I), cyanidin (II) and delphinidin (III), which differ only in the number of hydroxyl groups in the 2-phenyl nucleus:



I



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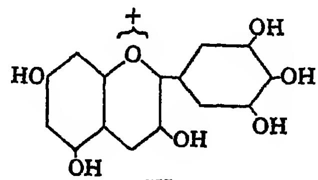
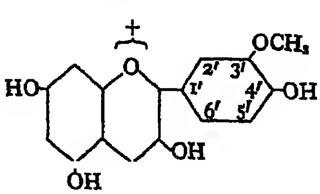


III

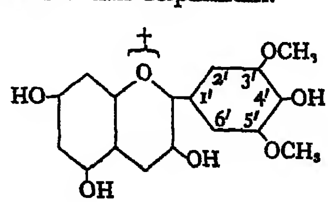
These substances, known as anthocyanidins, are the colour-producing part of the anthocyanin molecule. They do not occur free in nature, but in combination with one or two molecules of a sugar, this compound being an anthocyanin.

As shown, cyanidin has one more hydroxyl group in the molecule than pelargonidin, and delphinidin two more. This is one of the principal factors upon which variation in flower colour depends, as an increase in the number of oxygen atoms (in the form of —OH groups) causes a marked increase in blueness of tone. The scarlet pelargonium, deep red rose and purple delphinium are good examples of colours due to pelargonidin, cyanidin and delphinidin derivatives respectively.

A second variable involving structural difference in the anthocyanidin molecule concerns the 3'- and 5'-hydroxyl groups,[3] which may or may not be methylated, that is, have the hydrogen atom of the hydroxyl group replaced by a methyl group, —$CH_3$. Other conditions being the same, methylated anthocyanins or anthocyanidins are redder than the corresponding unmethylated types, e.g. peonidin (IV) is redder than cyanidin and malvidin (V) is redder than delphinidin.



IV



V

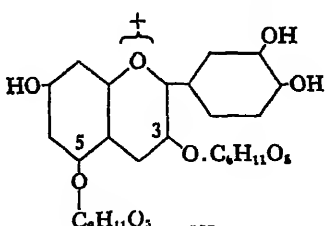
[1] Two series of papers in the *J. chem. Soc.* "A synthesis of pyrylium salts of anthocyanidin type", parts I–XXII, 1922–34; "Experiments on the synthesis of anthocyanins", parts I–XXVI, 1926–34.

[2] A series of papers in *Helv. Chim. Acta*, "Über Pflanzenfarbstoffe", 1927–32.

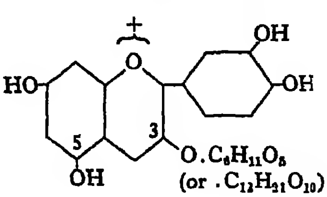
[3] It affects three groups in the anthocyanin hirsutin, found in *Primula hirsuta*, which is a 3':5':7-*O*-trimethyl delphinidin derivative. This, however, only occurs in some four species.

Reference to formulae I, II and III shows that there is one methylated derivative of cyanidin—3′-*O*-methyl cyanidin (peonidin), and two of delphinidin, the 3′-*O*-methyl derivative (petunidin) and the 3′:5′: *O*-dimethyl derivative (malvidin).

As mentioned above, the anthocyanins are compounds involving one or two molecules of a monose. Of these sugar molecules, one is always attached at the 3-position. If a second sugar molecule is present, it may either be attached directly to the first one or be linked with the anthocyanidin at position 5-. Hence there are two classes of glycosides: (*a*) those with a monose or a biose attached at position 3-, (*b*) and those with monose units at both 3- and 5-. These two classes differ in colour, the 3:5-dimonosides (VI) being bluer than the corresponding 3-type (VII).




VI




VII

For the terminology employed in describing these glycosides see Robinson & Robinson (1932).

So far three factors influencing the colour of anthocyanins have been dealt with, namely:

(i) the number of substituent hydroxyl groups in the anthocyanidin molecule,
(ii) the methylation of hydroxyl groups,
(iii) the position of attachment of sugar molecules.

Combinations of these three give rise to twelve anthocyanins each slightly different in colour, but together covering a wide range from scarlet to purple. The three factors are all dependent upon structural changes in the anthocyanin molecule, that is, the differences are internal. Conditions external to the molecule may also affect the colour of the anthocyanins. The most important of these conditions is a phenomenon known as copigmentation which will be referred to in connexion with the anthoxanthins.

Modification of flower colour can also be brought about by variation of a second external factor: the *p*H of the cell sap. The colour of pure natural or synthetic anthocyanins, all of which are indicators, varies from red to blue according to the *p*H of the solution, being blue at a high *p*H and red at a low one (see Robinson & Robinson, 1933). Comparisons of the colour of different anthocyanins should therefore be made in solutions of the same *p*H (Robertson & Robinson, 1929). The suggestion that many flower colour variations are due to changes in the *p*H of the cell sap was made by Willstätter & Everest (1913), Buxton (1932) and others. This was proved correct by Philip-Smith (1933) making use of the indicator nature of the anthocyanins themselves and by Scott-Moncrieff (1936) using a glass electrode. It should be emphasized that the values given by the latter direct method are not

those of the uncrushed petal, and are nearly always on the acid side of the neutral point; the recorded *p*H of blue flowers is in itself insufficient to account for the blueness of tone (Robinson & Robinson, 1933). Nevertheless, significant *p*H differences are found between red and blue flowers of genetically related plants containing the same anthocyanin. These differences are usually of the order of 0·5–1·0 *p*H (Scott-Moncrieff, 1936).

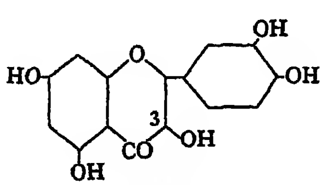
It has been suggested by Robinson & Robinson (1933) that the anomalous behaviour of many blue flowers in showing a cell sap *p*H of less than 7·0 is due to the existence of colloidal solutions of the anthocyanin, which are bluer than a true solution at the same *p*H.

### (2) *The anthoxanthins*

The anthoxanthins are sap-soluble pigments, usually glycosides, that range in colour from ivory to yellow. They are closely related chemically to the anthocyanins, though more varied with respect to the numbers and positions of substituent groups, and they fall into two classes, the flavones and flavonols. These classes differ in that the flavones have no substituent hydroxyl group at position 3-.

The anthoxanthins most commonly found in flowers are the flavonols quercetin (VIII, e.g. in *Bougainvillea*, Price & Robinson, 1937) and kaempferol (IX, e.g. in *Crocus*, Price *et al.* 1938) and the flavones apigenin (X, e.g. in *Antirrhinum*, Wheldale & Bassett, 1913) and luteolin.

In general, increase in the number of hydroxyl groups slightly intensifies the colour.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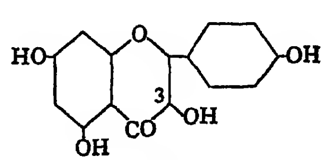
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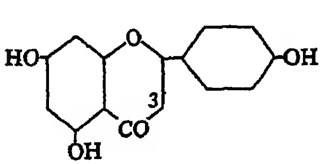
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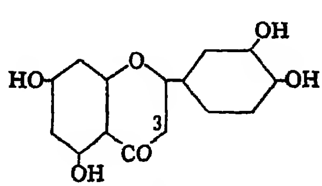

XI

There are four ways in which the anthoxanthins may be concerned in flower colour:

(*a*) In flowers which have no anthocyanin they may be directly responsible for some or all of the colour.

(*b*) When a yellow anthoxanthin occurs together with an anthocyanin, the resultant colour is a blend of the two.

(*c*) In the presence of anthocyanins, ivory anthoxanthins do not contribute independently to the colour, but they may do so indirectly by their "copigmenting" action. Copigments are substances which when present in the same solution as

an anthocyanin form weak additive complexes that are much bluer than the anthocyanin alone. Willstätter & Zollinger (1916) observed that the addition of tannin to a solution of malvidin 3-glucoside produced this effect. Robinson & Robinson (1931) and later Lawrence (1932) pointed out that such complexes play an important part in producing variations of flower colour. The anthoxanthins are not the only natural copigments; tannins and other unidentified substances also act in this way. Copigmentation varies in degree according to the nature of the anthocyanin; delphinidin derivatives are most readily copigmented and pelargonidin derivatives least so. It is also dependent upon the nature of the anthoxanthin, ivory anthoxanthins usually being the most effective.

(*d*) The structural similarity of the anthocyanins and anthoxanthins suggests that their syntheses in the plant may be correlated. Evidence from *Dahlia* (Lawrence & Scott-Moncrieff, 1935) shows that the two classes of substances are formed from the same starting materials which are probably limited in quantity. This results in competition between the two, and if most of the precursor is utilized in the synthesis of one pigment, then of necessity less of the other is produced. Thus the presence of much anthoxanthin may lead to almost complete suppression of anthocyanin, producing delicately flushed flowers. Further, if the anthoxanthin concerned is a copigment for the anthocyanin, then there is a modification of colour as well as of intensity.

### (3) *The carotinoids*

Although there are no combined chemical and genetical data on the inheritance of the non-sap-soluble flower pigments, some mention of them must be made. They comprise a number of yellow or orange substances, xanthophylls and carotins, which are carried by plastids or are dissolved in oils (Möbius, 1885). In the absence of anthocyanins they are either solely responsible for flower colour, or are supplemented by yellow anthoxanthins. In the presence of anthocyanins the colour is a blend of the two.

To summarize the position, variation in flower colour may be brought about in the ways shown in Table I. Changes are shown in one direction only; the reverse may be inferred.

Table I

| | |
|---|---|
| Anthocyanins: | |
| i. Increase in number of hydroxyl groups | Increased blueness |
| ii. Alteration from 3- to 3:5-sugar types | Increased blueness |
| iii. Methylation of one or more hydroxyl groups | Increased redness |
| iv. Increase in *p*H of the cell sap | Increased blueness |
| v. Copigmentation (anthoxanthins, tannins, etc.) | Increased blueness |
| Anthoxanthins: | |
| vi. Increase in number of hydroxyl groups | Increased yellowness<br>Alteration of background<br>Change in copigment effect |
| vii. Interaction of anthocyanins and anthoxanthins | Partial suppression of one or both types |
| Plastid pigments: | |
| viii. Alteration in nature of plastid pigment | Yellow ⇌ orange<br>Alteration of background |

## II. THE INHERITANCE OF COLOUR VARIATIONS

It is clear from the preceding account that the visual method of classifying flower colour variations is analytically inadequate. A change from red to blue, for example, can be brought about in five different ways. Should the plant under examination make use of two or more of these ways, as is often found, the inheritance may be confusing or even completely misleading. In addition to facilitating genetical classification, a knowledge of the chemistry of the substances concerned constitutes a first step towards an understanding of the mechanism of gene action. The question of the biogenesis of the anthocyanins, anthoxanthins and related substances necessarily involves the recognition of the successive stages of their synthesis, and one method of attack is by the separation of the effects of the genes controlling these stages. Genetics and biochemistry must go hand in hand, since any information bearing on synthesis *in vivo* means a step forward in the interpretation of genetical data, and conversely.

The first to realize this was Wheldale (1914) who attempted to analyse the pigments in various genotypes of *Antirrhinum majus*. Unfortunately little was known at that time of the chemistry of the anthocyanins, and perhaps the main outcome of Wheldale's work was to draw attention to the potentialities in this field. Later Scott-Moncrieff, at the suggestion of Haldane, began a series of combined chemical and genetical studies of flower colour variation, and it is from these studies that the greater part of our knowledge of the subject has been derived (see Scott-Moncrieff, 1936; Lawrence & Scott-Moncrieff, 1935; Beale *et al.* 1939; Lawrence *et al.* 1939; Haldane, 1935, 1937).

For convenience of presentation, we shall consider the action of genes involved in pigment production under two heads—"qualitative" and "quantitative". The former refers to the production or non-production of a pigment, and the latter to difference in the amount of pigment. The distinction is obviously arbitrary, and breaks down in extreme cases. Modification of chemical structure is, of course, regarded as a qualitative change.

### (1) *The role of genes in pigment production: qualitative effects*

In many garden plants there are coloured and white-flowered varieties. The white forms usually originate as mutants from the coloured, and differ from them in respect of a single gene. Thus the hybrid from crossing the coloured and white-flowered forms of *Phaseolus multiflorus* resembles the coloured parent (Tschermak, 1904). On self-fertilization of this hybrid, coloured and white-flowered forms segregate in the ratio 3 : 1. The presence or absence of anthocyanin is here determined by a single gene, which in the recessive condition is apparently inactive. In like manner, the production of anthoxanthins may be controlled by a single gene. Magenta flowers of *Primula acaulis* differ from the red in that the former contain large amounts of an anthoxanthin copigment not present in the latter. This difference has been shown to be due to a single gene (Buxton, 1932; Scott-Moncrieff, 1932, 1936).

The formation of the carotinoid or so-called "plastid" pigments is controlled in the same way. In *Cheiranthus Cheiri* (Scott-Moncrieff, 1936) the presence or absence of a deep yellow non-sap-soluble pigment is governed by a gene **Y**. In flowers containing anthocyanin the yellow pigment has a strong background effect, and the flower colour is brown or orange as compared with the purple or pink of the plants recessive for **Y**.

These examples deal with dominant genes which give rise to the formation of some pigment. In certain cases, however, it is the recessive form which is pigmented, while the dominant allelomorph is apparently negative. For example, in *Primula sinensis* (de Winton & Haldane, 1933) the white-flowered type is dominant to the coloured, and in *Papaver Rhoeas*, the production of an anthoxanthin copigment is a recessive character (Scott-Moncrieff, 1936).

In an account of the genetics of *Zea Mays*, Eyster (1934) lists thirteen groups of complementary genes which affect chlorophyll production. One of these groups includes eleven recessive genes giving rise to albino seedlings containing at most only traces of chlorophyll. That is, eleven dominant genes are necessary for the normal production of chlorophyll and the carotinoid pigments. There is also a second group of seven dominant genes which are necessary for the production of chlorophyll alone. In these two groups alone, then, eighteen dominant genes had been recognized in 1934 (and others have since been found) which are necessary for the production of chlorophyll. These genes probably act in series and control successive stages in the synthesis of chlorophyll (see Haldane, 1937). The identification of dominant genes is of course only possible when mutant recessives arise. In the case of the flower pigments no instance is known in which so many genes have been identified, but there are several plants where more than one gene is necessary for the production of anthocyanins or anthoxanthins.

In *Cheiranthus Cheiri* (Scott-Moncrieff, 1936) and *Lathyrus odoratus* (Bateson *et al.* 1905) two complementary genes are necessary for anthocyanin formation. Similarly, in *L. odoratus*, an anthoxanthin copigment is produced only when the genes **K** and **M** are present (Beale *et al.* 1939). The position in *Pharbitis nil* (Hagiwara, 1932) is more interesting. Four complementary genes **Ca**, **C**, **A** and **R** are necessary for anthocyanin formation. The flowers of all plants recessive for **Ca** are dead white and contain no anthoxanthin. The dominant gene **Ca** produces some anthoxanthin; **C** in the presence of **Ca** produces much anthoxanthin and **R** and **A** together with **Ca** and **C** produce anthocyanin. In addition, dominant whites are known (Hagiwara, 1929).

*Matthiola incana* (Saunders, 1928) and *Linum usitatissimum* (Tammes, 1922; Searle, 1926) carry respectively two and three complementary genes for anthocyanin formation. In both cases the effect of these genes alone is sufficient to produce only an extremely light pink, hardly distinguishable to the eye from white. An additional gene (or genes) is necessary for the development of coloured phenotypes of average intensity.

### (2) *The role of genes in pigment production: quantitative effects*

A gene is said to be dominant when it produces the same phenotypic effect in both the homozygous and the heterozygous conditions. Such a gene has been mentioned in the case of *Phaseolus multiflorus*, where the heterozygote from coloured × white resembles the homozygous coloured parent. There are numerous other examples. However, in *Mirabilis jalapa* the $F_1$ from crossing crimson × yellow is intermediate, i.e. it has orange-red flowers and segregates 1 crimson : 2 orange-red : 1 yellow in $F_2$ (Marryat, 1909). This quantitative difference between the homozygous and heterozygous forms indicates that one gene of the kind segregating does not have the same effect as two. Such characters are said to be incompletely dominant. A theory to account for them, advanced by Goldschmidt (1927, 1938), is that differences in rates of reaction can determine whether dominance is complete or incomplete. If this is so, the phenotypic expression of the gene concerned should be dependent upon the temperature, since temperature is one of the factors controlling reaction velocity. The amount of an end-product, as well as its rate of formation, should be greater at a higher temperature provided the temperature coefficient of competing processes is not higher than that of the reactions leading to the end-product under consideration. No quantitative work has yet been done on flower pigments, but cases are known where modification follows temperature variation.

Klebs (1906) observed a variation in the flower colour of *Campanula Trachelium* from white, in heated greenhouses, through pale blue to deep blue in the cold. In *Primula sinensis* (de Winton, private communication) the effect of a dominant gene inhibiting anthocyanin formation in the petals is incomplete in phenotypes containing anthocyanin in the stigma. Such plants show an increase in the amount of anthocyanin in the petals when kept at a lower temperature. The possibility of a rise in temperature causing an increase in the amount of anthocyanin is not realized in these two examples. Klebs considered this to be due to the fact that growth at high temperatures is so rapid that anthocyanin intermediates are not available in sufficient quantity. However, Kuilman (1930, quoted by Karstens, 1938) found that at 5° C. seedlings of *Fagopyrum esculentum* develop an anthocyanin, cyanidin 3-glycoside (Karstens, 1938), slowly and after some time the amount of pigment remains constant. *At higher temperatures* (25°, 30°) *the anthocyanin appears more quickly*, though the quantity is less than at lower temperatures. By analogy with all genetically analysed cases, this process must be gene controlled. It shows, then, that the expression of the genes governing anthocyanin formation is dependent upon reaction velocity. Probably the majority of quantitative differences in gene-controlled processes—incomplete dominance, intensifying and diluting effects—can be explained in this way.

In diploid plants incomplete dominance is found less frequently than in polyploids. In the latter more than two of each gene may be present, and additive effects are common. Johnson & Miller (1938) found that the amounts of total carotinoids and of $\beta$-carotene in the endosperm of *Zea Mays* is approximately in

direct proportion to the number of "dominant" **Y** genes. The vitamin A activity is also in close agreement (Mangelsdorf & Fraps, 1931). The results are shown in Table II.

Table II

| Endosperm genotype | Total carotinoids | | β-carotene | | Relative vitamin activity |
|---|---|---|---|---|---|
| | % | Ratio | % | Ratio | |
| YYY | 0·000465 | 3·3 | 0·000131 | 3·1 | 7·50 |
| YYy | 0·000282 | 2·0 | 0·000079 | 1·9 | 5·00 |
| Yyy | 0·000139 | 1·0 | 0·000042 | 1·0 | 2·25 |
| yyy | 0·000042 | 0·3 | 0·000011 | 0·3 | 0·05 |

In flowers of *Dahlia variabilis* the effect of the gene **A** governing anthocyanin formation is additive, the flowers of an **AAAA** genotype containing more anthocyanin than those of **AAAa**, **AAAa** more than **AAaa** and so on (Lawrence & Scott-Moncrieff, 1935).

Many genes have been identified whose end-effects are purely quantitative; they alter the amount of pigment produced. For instance in *Papaver Rhoeas*, the genes **C** and **B** increase the amount of anthocyanin (Scott-Moncrieff, 1936). In *Primula sinensis* the gene **I** has the opposite effect; it reduces the amount of anthocyanin (Scott-Moncrieff, 1936). In addition there are intensifying and diluting genes whose action is not effective over the whole petal, but is restricted to certain areas, giving rise to patterns.

Another factor causing quantitative differences in pigment production is interaction. In *Primula sinensis* (Scott-Moncrieff, 1936) the copigmented magenta flowers have less anthocyanin than the corresponding uncopigmented red flowers, which contain no anthoxanthin. The product of the gene **B** controlling anthoxanthin formation thus interacts with the product of the genes governing anthocyanin formation and the change from the dominant (**B**) to the recessive (**b**) results indirectly in an increase in the amount of anthocyanin.

### (3) *Gene control of pigment modification*

The previous two sections deal with chemical processes about which we know little or nothing, except that they terminate in the formation of an anthocyanin or anthoxanthin. We shall now consider examples of gene action in which we do know what the gene is doing, though not yet how it does it.

In the first place, the number of hydroxyl groups in the 2-phenyl ring of the anthocyanidin molecule is determined genetically. Wit (1937) found that in *Callistephus hortensis* the change pelargonidin → cyanidin → delphinidin is controlled by three multiple allelomorphs. Thus if a homozygous plant containing cyanidin 3-glycoside is crossed with one containing pelargonidin 3-glycoside, the progeny all contain cyanidin 3-glycoside and on self-fertilisation give three plants

with cyanidin derivatives to one with the pelargonidin derivative. Similarly, a cross between plants with delphinidin and cyanidin derivatives respectively yields progeny containing the delphinidin anthocyanin, and both parental types segregate in $F_2$.

There are two principal colour classes in *Verbena hybrida* (Beale & Scott-Moncrieff, unpublished). The flowers of these are pigmented with derivatives of pelargonidin and delphinidin respectively, the production of which is determined by three allelomorphs. In *Streptocarpus* (Lawrence *et al.* 1939) two genes **O** and **R** determine the state of oxidation of the anthocyanin. Plants recessive for **O** and **R** produce pelargonidin derivatives, **Ro** produce cyanidin derivatives and **RO** or **rO** produce delphinidin derivatives. The situation in *Lathyrus odoratus* (Beale *et al.* 1939) is the same as in *Streptocarpus*. A further example is *Primula sinensis*. Here a gene **K** is responsible for the production of a delphinidin derivative, malvidin 3-galactoside, while its recessive gives rise to pelargonidin 3-glycoside.[1]

As we shall show, there is good reason to suppose that the production of delphinidin involves one more stage than that of cyanidin, and that this stage is one of oxidation. Similarly the production of pelargonidin requires one more stage than that of cyanidin, in this case a reduction. Hence the genes referred to are bringing about oxidation or reduction processes. As in the more general problem of pigment production, we find that these processes are not always carried to completion. In *Streptocarpus* the delphinidin and the pelargonidin derivatives sometimes contain traces of cyanidin derivatives (Lawrence *et al.* unpublished) and in *Verbena* both the pelargonidin and delphinidin derivatives may contain small amounts of anthocyanin derived from cyanidin.

A second type of chemical change which is genetically controlled is that of glycoside formation. In *Verbena* the anthocyanins are either 3-monosides or 3:5-dimonosides and the difference between these two classes is genetically determined. Again, in *Streptocarpus* there are two main glycosidal types, the 3:5-dimonoside and a second which consists of a mixture of 3:5-dimonoside with 3-pentose-glycoside. A dominant gene **D** gives rise to 3:5-dimonoside and its recessive **d** to the mixtures of 3-pentose-glycoside and 3:5-dimonoside. This situation is more complex than that in *Verbena*, as recent results indicate that several genes, all of which are hypostatic to **D**, are concerned in the production of the mixtures.

In *Verbena* the action of the gene determining the glycosidal type must be to bring about union with a hexose molecule at position 5 or conversely to remove a hexose residue at this point. Here also examples are found where the reaction is not completed and mixtures of 3-monoside and 3:5-dimonoside result.

A third chemical factor causing colour variation in the anthocyanins is methylation of hydroxyl groups. Evidence from *Streptocarpus* (Lawrence *et al.* unpublished) shows that this is genetically controlled, but no simple segregation of methylated and unmethylated types has yet been observed, so more than one gene pair is responsible. Nevertheless, it is worth mentioning that incompleteness of methylation

[1] Probably galactoside, but the sugar has not yet been identified.

is very common; for example, mixtures of malvin with small amounts of petunin have been found in *Lathyrus odoratus* (Beale *et al.* 1939), while in *Streptocarpus* comparable plants from the same family containing malvidin and peonidin derivatives respectively show a wide difference in the extent to which methylation is incomplete. In plants carrying the methylating gene or genes, unmethylated anthocyanins of the delphinidin series occur only in small amounts (*ca.* 1–5 %), but in the cyanidin series it is usual to find as much as 50% of unmethylated anthocyanin.

The extent of methylation is correlated not only with the degree of oxidation, but also with the glycosidal type. The 3-pentose-glycosides of the delphinidin series usually contain small amounts of anthocyanins derived from petunidin or delphinidin which are not present in the 3:5-dimonosides. The same applies in the cyanidin series where methylation is more complete in the 3:5-dimonosides than in the 3-pentose-glycosides. Perhaps the most interesting interaction of this type was found by Beale *et al.* (1939) in *Lathyrus odoratus.* In the delphinidin series, the anthocyanins are fully methylated when the copigment (quercetin) is present, but when this is absent methylation is incomplete and mixtures of malvin, petunin and delphin are found.

Robinson & Robinson (1931) devised a set of qualitative tests by which rapid and accurate identification of anthocyanins is possible with small amounts of material. These tests were soon applied to the study of the inheritance of flower colour variations. Unfortunately no such tests are available for the identification of anthoxanthins, for which tedious large-scale processes are still necessary. For this reason there are few precise data available concerning the inheritance of the flavones and flavonols.

Wheldale & Bassett (1913) found that a gene **Y** produces luteolin in the lips and apigenin in the tube of *Antirrhinum* flowers and a second gene **I** modifies the luteolin to apigenin. However, recent preliminary work (Price, unpublished) has cast doubt on the validity of the identification of the yellow pigment as luteolin. It is possible that the substance is a chalkone.

All cyanic flowers of the garden *Streptocarpus* contain anthoxanthins, but some are copigmented and others not. The difference between the copigmented and uncopigmented forms is determined by a single gene which apparently modifies the structure of the noncopigmenting anthoxanthin in such a manner that it becomes capable of forming an additive complex with the anthocyanin. A similar situation is found in certain genotypes of *Primula sinensis*, which contain large amounts of an anthoxanthin that does not act as a copigment.

Two further identifications of anthoxanthins in genetic material are noteworthy. First, Beale *et al.* (1939) identified quercetin, accompanied by a small amount of kaempferol, in the flowers of *Lathyrus odoratus*. The nature of the flavonol is unaffected by modification of the anthocyanin. Secondly, Sando & Bartlett (1922) isolated quercetin, as the glucoside iso-quercitrin, and later the closely related cyanidin 3-monoglucoside (Sando *et al.* 1935) from husks of *Zea Mays*.

Gene control of *p*H differences in the cell sap of flowers has already been referred to. The cases known are *Primula sinensis*, *Primula acaulis*, *Papaver Rhoeas*,

*Tropaeolum majus* (Scott-Moncrieff, 1936), *Lathyrus odoratus* (Beale *et al.* 1939) and *Trifolium pratense* (Price & Williams, unpublished).

Two examples may be cited to illustrate the independent segregation of the genes whose action we have been considering, and to show how a wide range of flower colours results from the various combinations of only a few genes. Three of the major gene pairs governing flower colour in *Primula sinensis* are **K–k**, **B–b** and **R–r**. **K** gives rise to malvidin 3-galactoside and the recessive to the corresponding pelargonidin anthocyanin. **B** produces an anthoxanthin copigment, and **R** increases the acidity of the cell sap. On self-fertilization of a plant heterozygous for all three genes the following classes and ratios are obtained:

| | | |
|---|---|---|
| 27 | **KBR** | magenta; copigmented malvidin anthocyanin; more acid cell sap. |
| 9 | **KbR** | red; uncopigmented malvidin anthocyanin; more acid cell sap. |
| 9 | **KBr** | blue: copigmented malvidin anthocyanin: less acid cell sap. |
| 3 | **Kbr** | slaty; uncopigmented malvidin anthocyanin; less acid cell sap. |
| 9 | **kBR** | almost white; pelargonidin anthocyanin + anthoxanthin; more acid cell sap. |
| 3 | **kbR** | coral; pelargonidin anthocyanin, no anthoxanthin; more acid cell sap. |
| 3 | **kBr** | almost white; pelargonidin anthocyanin + anthoxanthin; less acid cell sap. |
| 1 | **kbr** | pale coral; pelargonidin anthocyanin, no anthoxanthin; less acid cell sap. |

Similarly with the five genes, **A** (necessary for anthocyanin production), **R**, **O**, **D** and **I** (producing anthoxanthin copigment) in *Streptocarpus*, we obtain eleven different colours, blue, purple, blue-mauve, mauve, bluish magenta, magenta, bluish rose, rose, pink, salmon and white, which segregate in normal Mendelian ratios from the appropriate crosses.

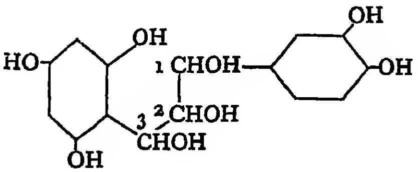
## III. THE BIOGENESIS OF THE ANTHOCYANINS AND ANTHOXANTHINS

The biogenesis or mechanism of synthesis of the anthocyanins has inspired numerous publications from 1682 to the present day. A good account of earlier theories is given by Onslow (1925). The possibility that there is a synthetical interrelation with the anthoxanthins was recognized by the same author (Wheldale, 1909) before the structural formulae of the anthocyanins had been worked out. She supposed (1911) that the anthocyanins were oxidation products of flavones or flavonols. When it was shown that the change from flavonol to anthocyanidin must involve reduction, not oxidation, the earlier theory was discarded in favour of a reduction process (for example, see Shibata, 1915).

Other theories, involving tannins, have also been put forward, but the most important contribution is that due to Robinson (1934) who suggests that the anthocyanins, flavones, flavonols and the related catechins and flavanones may all be derived, in different ways, from the same intermediate. In other words, the production of the anthocyanins and anthoxanthins is parallel, not sequential. The hypothetical intermediate (XII) can be built up from two hexose units and one

triose unit by a series of aldol condensations, and dehydrations. Oxidation at carbon atom (1), dehydration between (2) and (3) and ring closure would give cyanidin.

Oxidation at carbon atom (3), followed by dehydration and ring closure, would give luteolin, and oxidation at (2) and (3), or at (1) and (3), and then ring closure would give the flavonol quercetin. Support for the parallel syntheses of anthocyanidins and anthoxanthins is furnished by genetical observations of the com-



XII

petition in development between the two classes of substances, particularly in *Dahlia variabilis* (Lawrence & Scott-Moncrieff, 1935; Robinson, 1936). Robinson's theory was put forward on the basis that the structural units are $C_6$–$C_3$–$C_6$ and that these residues have a state of oxidation comparable with that of a carbohydrate. If the aldol condensations take place in the direction $C_6 \rightarrow C_3 \rightarrow C_6$ and the $C_6$ groups become phenolic nuclei, the left-hand ring will have two and the right-hand ring three hydroxyl groups. This is found to be so in the majority of anthocyanins, flavonols and catechins, which contain catechol and phloroglucinol nuclei respectively. If this reasoning is justified, it follows that the 2-phenyl ring of the anthocyanins and flavonols represents the hexose unit that first became attached to the three-carbon fragment and it might be expected that this part alone would occur in nature. This is indeed the case, and the orientation of the hydroxyl groups in the benzene ring in such substances is commonly of the catechol type. This theory implies that cyanidin and quercetin are synthetically the simplest members of their classes and that pelargonidin and kaempferol or delphinidin and myricetin require an extra stage of reduction or oxidation respectively. A survey of the frequency of distribution (Lawrence *et al.* 1939) provides strong confirmation of this view.

Robinson & Robinson, by qualitative tests (1931, 1932, 1934) identified the anthocyanins in large numbers of flowers and fruits. These surveys were then extended to young leaves, in which the appearance of anthocyanin is only transient (Price & Sturgess, 1938) and to autumn leaves (Lawrence *et al.* 1938). The colour of flowers and fruits is known to be of importance to the organism in attracting insect pollinators and bird distributors respectively. Since the colour is altered by a change in the state of oxidation of the anthocyanidin, a mutant form containing an anthocyanin derived from a different anthocyanidin will be selected if the new colour is more advantageous to the plant than the old. In flowers and fruits therefore we may expect to find a greater variation in the nature of the anthocyanidin than in those parts of the plant where the anthocyanin has little or no value, and is not subjected to selection for colour. This was found to be so, as shown in Table III.

The selection of one anthocyanin type in preference to another is shown by the fact that over 90% of species whose flowers contain pelargonidin derivatives originate from tropical or other hot countries. In temperate climates, bees are the

Table III

| | Number of genera containing cyanidin derivatives expressed as a percentage of the total number of genera examined |
|---|---|
| | % |
| Autumn leaves | 95 |
| Young leaves | 93 |
| Permanently pigmented leaves | 76 |
| Fruits | 69 |
| Flowers | 50 |

commonest pollinators. Von Frisch (1937) found that bees are sensitive to blue but are red-blind and so a mutation which results in reduction to a pelargonidin derivative would be harmful to bee-pollinated species and would tend to be eliminated. On the other hand, a mutant form containing a delphinidin derivative is likely to be advantageous in cool climates and selection would operate in its favour. Conversely in hot climates the pollinating agents may be red-sensitive, e.g. butterflies. Then a mutation to the redder pelargonidin anthocyanin has a greater chance of survival. It is of interest that in a number of tropical plants whose flowers contain cyanidin derivatives, the anthocyanin is accompanied by a deep yellow carotinoid pigment, the mixture having much the same colour as pelargonin. Thus, when a mutation arises producing this advantageous effect it is selected regardless of the way in which the effect is brought about.

The flowers of the majority of "good" species are pigmented by one anthocyanin only, but there are a few cases in which mixtures are encountered. This is of interest in connexion with the theory of dominance put forward by Muller (1932). Muller supposes that dominance is developed by the selection of mutations which provide a margin of stability to the expression of the gene, insuring the organism against excessive variability of the character either by environmental or genetic influences. On this view, if a certain flower colour has a positive selective value, the production of the pigments concerned should take place under optimum conditions—conditions which not only allow the various stages in the synthesis to be completed, but also provide a "margin of safety" enabling the reactions to proceed normally in different environments. In all probability the decisive factor is the rate at which the reactions take place. Mutations increasing the velocity of the more important stages, such as that which differentiates delphinidin from cyanidin, should therefore be selected and in the majority of species or stable communities we should not expect to find mixed anthocyanins.

The simplest class of anthocyanin mixture is due to incomplete methylation; for example, *Geranium psilostemon* contains malvin together with a small amount of

petunin. More important are those in which the anthocyanins are derived from anthocyanidins with a different degree of oxidation. In these species we find mixtures of pelargonidin and cyanidin derivatives, and of delphinidin and cyanidin derivatives, but not mixtures based on pelargonidin and delphinidin. The cyanidin is usually, but not always, present in small quantities only. This is taken to mean that the production of both pelargonidin and delphinidin involves at least one more stage than that of cyanidin, and that this stage is not always carried to completion. It should be emphasized that there is no suggestion that cyanidin glycosides themselves are oxidized or reduced—these processes would take place at a stage prior to the actual formation of the anthocyanins. Phylogenetic classification of the flowers which had been examined gave further evidence in support of the hypothesis.

Recently, Bancroft & Rutzler (1938) have revived the hypothesis that anthocyanins are formed from anthoxanthins. They consider that this is so in some cases, but that in others the leuco-anthocyanins, as suggested by Robinson (1936) may be the precursors. The leuco-anthocyanins are colourless substances which on treatment with acids are converted into anthocyanidins. The conversion of leuco-anthocyanins to anthocyanins may take place in the plant under certain circumstances, such as in the development of autumnal coloration, but it is doubtful whether it is often so. Only two examples are on record where the anthocyanidin from both the anthocyanin and the leuco-anthocyanin have been identified in the same part of a plant, namely the flowers of *Hydrangea hortensis* and the fruits of *Vitis heterophylla* (Lawrence *et al.* 1939). The former contained delphinidin pentoseglycoside and the latter malvidin 3:5-dimonoside, but the leuco-anthocyanin from both yielded cyanidin.

Bancroft & Rutzler assert that the anthocyanins in red autumn leaves of sumach, dogwood and barberry are due to reduction of flavones. This conclusion appears to be based solely upon the fact that the green leaves contain flavones but no leuco-anthocyanins; evidence which is far from convincing.

The isolation of cyanidin 3-glucoside and the corresponding quercetin 3-glucoside from *Zea Mays* led Sando to favour the possibility that anthocyanins are reduction products of flavonols, regardless of the fact that cyanidin and quercetin derivatives are by far the commonest representatives of their classes (Gisvold & Rogers, 1938). On this basis alone it would be surprising if they were not frequently found together. Sando stresses the fact that previously only free flavonols had been studied in connexion with anthocyanins, whereas he and his collaborators found agreement not only between the aglycones but also in the nature of the sugar residue. This agreement obviously does not help to distinguish between the various theories which have been put forward.

Keeble & Armstrong (1912*a*, 1912*b*) considered that anthocyanins are formed by the action of an oxidase or peroxidase on a chromogen. That an oxidation process is involved in anthocyanin formation is quite clear from the results of Kuilman (1930), Karstens (1938) and others. Karstens showed that in *Fagopyrum esculentum* formation of anthocyanin depends upon the availability of carbohydrates, both in

respect of their quantity and distribution. He further showed that a photochemical reaction involving oxidation and a second oxidation process which can take place in the dark are the essential steps in anthocyanin synthesis. The fact that there appear to be two oxidation reactions need not be an objection to Robinson's hypothesis; as this author himself points out: "only the general direction of the process is suggested; the biochemical detail may be much more complex". The $C_3$ fragment may be the product of a degradation requiring oxidation.

## IV. DOMINANCE RELATIONSHIPS AND COMPETITION

The following is an extract from a paper by Moore (1910): "...if we take the view that life processes are chemical in their nature, we must of necessity accept the consequences which follow the application of chemical laws, and concede that such laws hold just as truly for chemical reactions within the living organism as for reactions *in vitro*...it seems reasonable that a clear explanation of the variations from Mendel's law of dominance lies...in the domain of physical chemistry." It is evident that the action of every gene must be capable of interpretation in chemical terms. Large numbers of chemical reactions, some more or less independent, others parallel or sequential, are proceeding simultaneously in every cell of a living organism. If we consider the possibilities for any single reaction, then a gene can be controlling this reaction in one of the following ways:

(1) In controlling supplies of the necessary reactants (such a gene might control a previous stage in the synthesis, or the permeability of the cells).

(2) In removing the reaction product—this would, for example, be necessary in a balanced reaction with an equilibrium point favouring the starting material.

(3) In supplying an enzyme or other catalyst.

(4) In controlling conditions, such as *p*H, which if unfavourable may lower the reaction velocity or even completely inhibit the reaction. Some of these points have been more fully discussed by Haldane (1932).

In the previous sections, the effects of genes involved in pigment production were separated for convenience into qualitative and quantitative. This distinction however is clearly an arbitrary one, since some qualitative differences may be straightforward presence and absence, e.g. of an enzyme, while others may be due to interaction. Many qualitative differences also arise from interaction, but in other cases both allelomorphs may act in the same way to a different degree, particularly by influencing reaction rates to different extents.

Dominance or recessiveness of a character is determined by the resemblance of the heterozygote to one or other of the homozygous forms. As Goldschmidt (1938) points out, dominance strictly is not an attribute of the gene, but is a phenotypic result of the action of the gene in relation to its genetic and external environments. For example, the action of the "dominant white" gene in *Primula sinensis* varies according to whether it is associated with the genes for "green stigma" or "red stigma" and also, as we have already seen, it varies with the temperature. It is by the consideration of reaction rates and competition that the various aspects of

dominance relationships are best co-ordinated. Strong support for this view is found in the modification and sometimes reversal of dominance by changes in temperature, which we know may profoundly influence reaction velocities (see Goldschmit, 1938). Dominance is thus determined by the reaction rate in the heterozygote relative to the rates in the two homozygotes. We shall now see how this idea is in keeping with the data on pigment inheritance.

Examples of complete and incomplete dominance in the production of plastid pigments, anthoxanthins and anthocyanins have already been mentioned. Complete dominance is the usual state of affairs, and in such cases heterozygotes certainly bear a close resemblance to the dominant forms, though it has not yet been established by quantitative comparisons that dominance is ever 100% complete. Nevertheless, the chain of reactions must proceed at a rate which does not vary greatly with a change in dosage of the active allelomorph.

As would be expected, it is impossible to lay down definite rules concerning the dominance or recessiveness of reactions leading to structural modification of the pigment molecule, but a higher state of oxidation is usually dominant to a lower. Thus the delphinidin pigment types in *Streptocarpus* are dominant to cyanidin, though the oxidation process is sometimes incomplete (see p. 44). This is best understood in relation to the rate at which oxidation takes place and to the competition between the oxidation process and the normal reactions leading to the formation of cyanidin. The pelargonidin types in *Streptocarpus* are much paler than, and recessive to, the cyanidin types. The rate of formation of the anthocyanin is evidently slowed down by the introduction of the reduction process so that the anthocyanin is produced in smaller amount than the corresponding cyanidin derivative. Moreover, cyanidin derivatives are also present, so the reduction is not carried to completion. It is not surprising then that reduction in the heterozygote cannot compete with the normal reactions leading to cyanidin, with the result that pelargonidin forms are recessive.

In species which have only the pelargonidin and delphinidin series of anthocyanins, e.g. *Verbena* and *Primula sinensis*, the oxidation and reduction processes are in competition with one another. Where two processes, $A$ and $B$, are in competition for an intermediate a change of conditions affecting reaction velocity may lead to partial or complete suppression of the end-product of $A$, with consequent increased formation of the end-product of $B$. In extreme cases the process $B$ may take place so slowly that in normal organisms its effect is unrecognizable. But when one of the genes controlling $B$ mutates to a more efficient condition, an entirely new substance or character—the end-product of $B$—appears. The same result could be achieved by a mutation lowering the rate of $A$. In *Primula sinensis* delphinidin derivatives are as a rule completely dominant to pelargonidin. Oxidation evidently proceeds at a much greater rate than reduction, so that the heterozygote contains no (detectable) pelargonidin. As in *Streptocarpus*, the flowers containing pelargonidin are very pale. However, there is a mutant gene known as "Dazzler" (**Dz**) which increases the production of pelargonidin. As a result, the flowers of **k Dz** forms are deep salmon and contain a much larger amount of pelargonidin 3-monoside than those

of **k dz**. The **K Dz** forms contain malvidin 3-monoside mixed with a smaller amount of pelargonidin 3-monoside. This is evidently due to an acceleration of the reduction process to a point where it competes on only slightly less than equal terms with the oxidation. That it does *not* completely "block" the oxidation process is not surprising in view of the fact that **k** plants heterozygous for **Dz** are intermediate in intensity between the two homozygotes and **K** plants heterozygous for **Dz** are usually indistinguishable *from* **K dz**. Moreover, **Dz** interacts with **B**, the gene governing anthoxanthin production, and the suppressing effect of **B** on **k Dz** forms is greater than in **K dz**, supporting the idea that the reaction rate determined by **Dz** is less than that determined by **K**.

The somewhat similar situation with regard to the anthocyanins in *Papaver Rhoeas* can be explained along the same lines.

At an early stage of this work, when less information was available, Scott-Moncrieff (1936) found it desirable to ascribe the behaviour of the "Dazzler" gene, and its analogues in *Papaver*, to a "specific" pigmentation process, though it is evident that even at that time certain contradictions between dependence and independence were inherent in this view. The idea of competitive processes outlined here does away with the necessity for postulating "specific" pigmentation, i.e. wholly independent processes to account for supposedly exceptional cases.

In *Verbena* there is little or no difference in intensity between the pelargonidin and delphinidin series, and competition between oxidation and reduction processes is presumably more equally balanced. This results in flowers of some plants containing mixtures of pelargonidin and delphinidin derivatives.

The functionally tetraploid *Dahlia variabilis* furnishes an excellent example of competition. The inheritance of flower colour in this plant was analysed semi-quantitatively by Lawrence & Scott-Moncrieff (1935). There are four principal genes governing flower colour: **A** and **B**, both of which produce anthocyanin, **I** which gives rise to the flavone apigenin, and **Y** which produces a deep yellow substance. This yellow pigment has not yet been identified. Schmid and his collaborators (1928, 1932, 1933) consider it is an isomer of apigenin, but work in progress (Price, unpublished) does not support this, though showing it to be closely related. The products of the genes **A**, **B**, **I** and **Y** interact with one another, and these genes have different competitive values. Moreover, they appear to control to different extents the quantity of "raw material" available for pigment production. This suggests that the intermediate (*S*), which the pigment genes are drawing upon, is preceded by a balanced reaction which is competing with other processes. The amount of "*S*" which is formed and converted to anthocyanin or anthoxanthin would then be dependent on the rate at which "*S*" was utilized. With the introduction of more pigment genes, the rate of utilization may be increased and consequently more may become available. This is evidently the case in *Primula sinensis* also, where processes controlled by **K** and **B** use up the intermediate more rapidly than when **K** is acting alone. As a result, the suppression of anthocyanin in **KB** plants, as compared with **Kb** where anthoxanthin is absent, is not in proportion to the increase in anthoxanthin content. In *Dahlia*, however, there is an upper limit

to the amount of intermediate available, and accumulation of the pigment genes **A**, **B**, **I** and **Y** cannot increase the total pigment production beyond a certain point. The genes **Y** and **B** appear to be completely dominant in the simplex condition, which of course means that one **B** or **Y** gene can utilize all the available intermediate. That it is the limitation of the quantity of intermediate which causes simplex **Y** and **B** to be dominant is shown by their interaction, which is dependent upon dosage.

**A** is cumulative up to quadriplex, but **I** appears to reach its maximum of production when duplex. The main features of the competition between the processes controlled by these genes are as follows. **Y** strongly suppresses the effects of **I** and **A**; for example, $\mathbf{Y}_1\mathbf{I}_3$ plants[1] contain a little apigenin, but $\mathbf{Y}_2\mathbf{I}_2$ contain none. Similarly $\mathbf{A}_1\mathbf{Y}_1$ plants contain little or no anthocyanin while $\mathbf{A}_4\mathbf{Y}_1$ have a small amount. The competitive value of **B** is greater than that of **I** or **A**, consequently in $\mathbf{B}_1\mathbf{Y}_1$ plants there is an appreciable amount of anthocyanin, though so far as one can tell, less of it than of the yellow substance. In $\mathbf{B}_1\mathbf{Y}_2$ plants the anthocyanin intensity is reduced. **I** interacts strongly with **A**, but almost complete suppression of anthocyanin formation occurs only in such extreme cases as $\mathbf{A}_1\mathbf{I}_3$ and $\mathbf{A}_1\mathbf{I}_4$. $\mathbf{I}_1$ is unable to suppress anthocyanin production by $\mathbf{B}_1$, but the intensity in $\mathbf{B}_1\mathbf{I}_4$ plants is considerably decreased as compared with $\mathbf{B}_1$. These quantitative variations are, then, the expression of a fairly straightforward competition. But there is one further point of importance. The genes **A** and **B** both produce anthocyanin, they are not complementary, and may have arisen from the same ancestral gene and control the same process. They are not specific for any particular anthocyanin, and no gene modifying the nature of the anthocyanin has yet been identified. Yet two anthocyanins, cyanin and pelargonin, may occur separately or together in *Dahlia* flowers. When the sum of the potential contributions or "activity values" of the pigment genes is below a certain critical value, the anthocyanin is pure cyanin, e.g. in all **A** and **AI** genotypes. But when this sum exceeds the critical value, as in all **AY** or **BY** genotypes, the anthocyanin is pelargonin or a mixture of pelargonin and cyanin. $\mathbf{B}_1$ plants contain cyanin, but $\mathbf{B}_2$ contain the pelargonin-cyanin mixture. Thus the nature of the anthocyanin seems to be determined by the combined competitive effects of all the pigment genes. Perhaps *Dahlia* is homozygous for a gene or genes governing reduction, which is unable to proceed satisfactorily under all conditions. It is hoped that some explanation of this curious state of affairs will be forthcoming when the structure of the yellow pigment is known.

It will be seen from the preceding account that inherited chemical differences originate in two ways: first from gene action, i.e. the action of a gene in relation to that of its allelomorph, and secondly from gene interaction, i.e. the action of a gene in relation to that of non-homologous genes. These relationships are interdependent, and together they are the determinants of dominance, and sometimes of epistasy.

Bateson (1909) called genes which prevent others from manifesting their effects "epistatic" and the concealed genes "hypostatic". For instance, in *Cheiranthus*

[1] The abbreviated symbolism of Lawrence & Scott-Moncrieff for tetrasomic inheritance in *Dahlia* is used in this article. Genetic constitutions are denoted by dominant factors only.

*Cheiri* the presence of a yellow anthoxanthin is masked when accompanied by a deeper yellow carotinoid pigment. In many such cases, inability to recognize the expression of the hypostatic gene is due to the use of methods which lack precision. For example, in *Dahlia* the deep yellow pigment produced by **Y** is said to be epistatic to the apigenin produced by **I**. But the presence of apigenin can be recognized when chemical tests are used; the same applies to the anthoxanthin in *Cheiranthus*.

However, epistasy is often developmental in character; it may arise from gene interaction. The gene **Y** in *Dahlia* has a greater competitive value than **A**, and it may inhibit the production of anthocyanin by the latter: then, **A** does not manifest itself in the presence of **Y** and is said to be hypostatic to **Y**. Similarly in *Streptocarpus*, competition between oxidation and reduction processes is all in favour of the former, so that **O** is epistatic to **r**. In each of these examples, epistasy is due to the simultaneity of action of the genes.

Epistasy also arises from interference in a chain of reactions, and it would seem that the term epistatic could logically be applied to that member of a pair of complementary genes which is known to have priority of action. But this is not always easy to establish. There are eleven genes in *Zea* controlling the production of both chlorophyll and the carotinoids, and a second group of seven genes which controls the synthesis of chlorophyll only and is without direct effect on that of the carotinoids. We might infer that the first group of genes acts before the second group. Such an inference can be treated more rigorously in the case of the anthocyanins and anthoxanthins. We know from the competition between them that the syntheses of the two classes of substances are interrelated; they not only have a common precursor, but are competing for this precursor at the same time. To prove this last point let us suppose that they draw upon the precursor at different times. If the two competing processes are controlled by two non-allelomorphic genes, each of which is completely dominant in the absence of competition from the other dominant (such as **B** and **Y** in *Dahlia*, or **B** and **I** when the dosage of **I** is two or more), then one of these genes (that which acts first) should have the same competitive effect in the homozygous and heterozygous condition. But the result of competition between **B** and **Y** or **B** and **I** is dependent on the dosage of both. Therefore the synthesis of the two classes of substances does include, at some stage, a simultaneous competition for a common precursor.

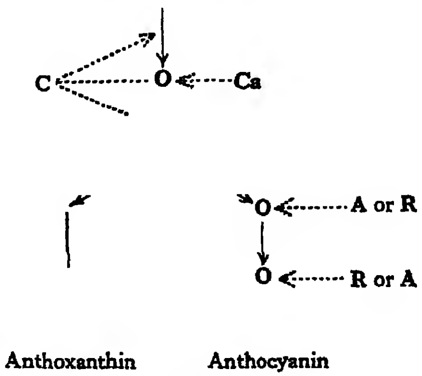



Fig. 1

In *Pharbitis nil* the gene **Ca** is essential for the production of both anthocyanin and anthoxanthin, therefore its effect is manifest at or before the separation of the two chains of syntheses. On the other hand, the genes **A** and **R**, both necessary for anthocyanin formation, do not directly influence the production of anthoxanthin. Therefore they become effective later than **Ca** and may be said to be hypostatic to **Ca**.

We do not know whether **A** precedes **R** or vice versa, nor do we know whether **C** precedes **Ca**, but we can outline roughly the sequence of events as in Fig. 1.

In *Streptocarpus* the extent of methylation is dependent on the state of oxidation and on the glycosidal type. Evidently the genes controlling methylation become operative later than **O**, **r** and **D**. Similarly in *Lathyrus*, the correlation between incomplete methylation and absence of anthoxanthin suggests that the methylation of the anthocyanin occurs after the divergence of the anthocyanin and anthoxanthin syntheses.

## V. SUMMARY

1. The principal flower colouring matters are the anthocyanins, anthoxanthins and carotinoids. Variation in colour depends upon the presence or absence of one or more of these substances, upon structural alterations in their molecules, changes in the *p*H of the cell sap, or quantitative changes affecting the amounts of pigment produced.

2. Pigment production is genetically controlled, and in a number of cases complementary genes are involved.

3. Variation in the amount of any pigment is also gene controlled. Such quantitative differences can be interpreted in terms of the velocity of the reactions involved in pigment production.

4. Modifications of the chemical structure of anthocyanins, including the state of oxidation, glycosidal type and probably the degree of methylation are each determined by simple gene relationships. In certain cases reactions are incomplete, giving rise to mixtures of anthocyanins.

5. The synthesis of anthocyanins in the plant is correlated with that of anthoxanthins. Some workers consider that the anthocyanins are formed from flavones or flavonols, but the most comprehensive theory, put forward by Robinson, postulates parallel formation of anthocyanin and anthoxanthin from the same intermediate. On this theory cyanidin and quercetin are synthetically the simplest members of their classes, others requiring additional stages of oxidation or reduction. The above view is supported by statistical analysis of the distribution of anthocyanins in flowers, fruits and leaves.

6. Heritable chemical differences result in the first place from gene action. They may be accentuated or minimized by gene interaction, which can modify dominance relationships and is sometimes the causal factor of epistasy. A study of gene interaction can also help in the determination of the sequence of gene action.

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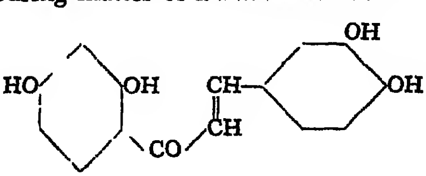
## ADDENDA

1. Since this article was written, a similar review has appeared on the "Genetics and Chemistry of Flower Colour Variation", by R. Scott-Moncrieff (*Ergebnisse der Enzymforschung*, 1939, **8**, 277).

2. The yellow colouring matter of *Dahlia variabilis* has been identified as the chalkone butein,



a substance structurally related to the anthocyanins and flavones, and belonging to the $C_6—C_3—C_6$ group (Price, *J. chem. Soc.* 1939, p. 1017). Thus, in *Dahlia* there are now three types of similarly constituted pigments instead of two, and competition between three genetically controlled lines of synthesis.

3. Hibbert (*J. Amer. chem. Soc.* 1939, **61**, 725) has discussed the formation of tannins and pigments in the light of recent work on lignin. In his opinion the theory that hexoses are precursors of the phenolic components of tannins, lignins and pigments does not provide a satisfactory solution of the problems of plant synthesis, and he puts forward the view that simpler substances, such as methylglyoxal or its dismutation isomers, may occur as intermediates. In effect, Hibbert's theory does not conflict with that of Robinson outlined in the present review, but carries the question a stage further back.

4. In a reply to Bancroft & Rutzler (1938), Robinson & Robinson (*J. Amer. chem. Soc.* 1939, **61**, 1605) again point out that they do not regard the production of anthocyanins from leuco-anthocyanins as the standard mechanism, but merely as an auxiliary process possibly operative in autumnal reddening and other special cases. G. M. Robinson (*J. Amer. chem. Soc.* 1939, **61**, 1606) has discussed those factors causing variations of flower colour which are not due to changes in the nature of the anthocyanin, e.g. the concentration of the anthocyanin relative to that of co-pigments. She also points out that chlorogenic acid may have some significance in relation to flower colour.

5. R. Harder (*Naturwissenschaften*, 1938, **26**, 713) describes the effect of temperature changes on the development of anthocyanins in several plants. In *Viola* and *Calceolaria*, in addition to the cases mentioned earlier in this article, increase in temperature results in a diminution in the intensity of anthocyanin pigmentation. But in *Petunia* the reverse is the case; the flowers of a variety which are self-coloured violet when grown at 35° C. have large white areas when grown at 25° C. and at 15° C. these white areas extend over nearly the whole of the flower. Not only anthocyanins, but also flavones and leuco-anthocyanins are inhibited in these white areas. A certain strain of *Dahlia variabilis* resembled *Petunia*, having yellow flowers when grown out of doors and red when grown in a hot-house at 30° C. This is probably a strain which is not pure recessive for genes controlling anthocyanin production, but one in which anthocyanin is normally suppressed by interaction with **Y** genes. In both *Petunia* and *Dahlia* Harder found that modifications arising from temperature differences are initiated during a sensitive period prior to the opening of the flower. In *Petunia* a decrease of light intensity also brings about increased anthocyanin production.

# UN APERÇU COMPARATIF DE LA GASTRULATION CHEZ LES CHORDÉS

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(*Received* 21 *April* 1939)

SOMMAIRE



## I. INTRODUCTION

QUELQUE soit l'œuf de Métazoaire que l'on envisage, il subit après la segmentation, un changement subit de forme, un remaniement profond de la répartition de ses masses cellulaires. Cette *gastrulation* précède toujours de peu l'apparition des organes primordiaux. On ne peut échapper à l'impression qu'elle en soit le véritable prélude.

Mais une telle préoccupation de considérer le développement comme un fait actuel dont les causes immédiates méritent d'être élucidées n'a pas toujours été le but des embryologistes. Les postdarwiniens considéraient plutôt l'Ontogénie en tant que reflet de la Phylogénèse. C'est Haeckel surtout qui s'est ingénié à édifier un système zoologique basé sur ce qui était considéré comme une récapitulation ancestrale. Suivant cette conception, la gastrulation, stade primitif et commun à tous les Métazoaires, doit représenter un état ancestral commun et primitif. C'est cette théorie de la *gastraea*, être didermique, muni d'une cavité centrale, l'*archentéron*, qui communique avec l'extérieur par un *blastopore* qui a servi de base fondamentale jusqu'à ces dix dernières années. Notons cependant que certains auteurs ne l'ont admis que sous de sérieuses réserves (cf. la 1ère édition du *Traité d'Embryologie* de A. Brachet, 1921).

La seconde idée maîtresse qui a longtemps dominé l'idée de la gastrulation des Chordés est celle de l'*entérocœlie* de O. & R. Hertwig (1881). D'après cette notion, chez certains Invertébrés et chez tous les Chordés, le mésoderme se forme par évagination de deux poches cœlomiques à partir de la paroi archentérique.

Enfin chez les Vertébrés encore, la troisième clef de la gastrulation était la *concrescence* de His (1873): le blastopore se fermerait par une véritable suture médiane des deux moitiés du corps.

Pour His, qui se basait sur les aspects de la gastrulation des Sélaciens et des Téléostéens, tout le corps embryonnaire se serait constitué par concrescence. Mais ultérieurement, les expériences d'électrolyse de Kopsch (1904) sur l'œuf de Truite ont fait accepter généralement la notion d'une concrescence restreinte au tronc et à la queue, la région céphalique restant unitaire; ce qui semblait d'ailleurs correspondre à l'interprétation que l'on donnait de l'asyntaxie blastoporale ("spina bifida") des Amphibiens (cf. O. Hertwig, 1892). Il est juste de noter que certains auteurs comme Morgan (1893, 1895) et Sumner (1904), étudiant la morphogénèse des Téléostéens, refusèrent leur crédit à toute idée de concrescence, sous quelque forme que ce soit. Mais une telle attitude n'était qu'exceptionnelle.

Une quatrième notion ayant joué un grand rôle également dans l'étude de la gastrulation est celle des "*centres de croissance*". Due surtout à Assheton (1895, 1905, 1916), elle suppose que certaines parties du corps seraient édifiées particulièrement par la croissance intensive de certains "centres" au niveau de la gastrula (voir aussi Veit, 1923). C'est d'elle que dérivent les distinctions en acro-, céphalo-, notogénèse, défendues surtout par Hubrecht (1890) et Brachet (1914, 1921).

Notons enfin le souci qu'avaient Hubrecht (1890) et Keibel (1900, 1905), spécialistes du développement des Mammifères, de ne considérer la gastrulation que comme la formation d'un *embryon didermique*, et de retrouver ce stade chez toutes les formes.

En dépit de ces idées directrices, le résultat était médiocre. Les controverses étaient plus nombreuses que les faits établis et seuls les spécialistes pouvaient s'imaginer comprendre la gastrulation. Ce qui était en défaut, c'était la méthode d'investigation: l'étude des "stades" sur coupes sériées. L'usage exclusif de cette méthode, beaucoup trop statique, ne peut convenir au dynamisme gastruléen. Le caractère cinétique de la gastrulation était cependant soupçonné. Mais le seul examen des coupes sériées ne pouvait décider de ce qui revenait éventuellement aux déplacements des cellules, à leur regroupement sur place ("délamination") ou à la prolifération.

L'examen de l'œuf vivant, muni de repères, sous forme de marques colorées locales avait été tenté dès 1911 et 1912 par Goodale et reprise plus tard par Smith (1914, 1922); mais c'est à Vogt (1925, 1929*a*) que revient le mérite d'imaginer une technique suffisamment maniable et de l'avoir exploité systématiquement sur les œufs des Amphibiens. Son mémoire fondamental (précédé de notes préliminaires en 1922, 1923, 1924) vint bouleverser de fond en comble nos notions sur la gastrulation des Vertébrés. Chose assez caractéristique, l'interprétation des aspects microscopiques, si pénible auparavant, devient évidente depuis que l'on connaît, par

l'étude des marques colorées, les voies des déplacements cellulaires; les deux méthodes se complètent.

Chez les Oiseaux, Gräper avait dès 1912 mis une méthode au point permettant de cinématographier des germes préalablement colorés au rouge neutre et d'y suivre les déplacements des particules. Les résultats en paraissent en 1929 en même temps qu'un mémoire de Wetzel qui étudie l'application des marques colorées localisées sur l'embryon de Poulet. A côté de résultats concordants, il subsiste toutefois des contradictions entre les deux auteurs, surtout dans leurs interprétations (cf. Gräper, 1930; Wetzel, 1931).

Dans les années qui suivent, les marques colorées sont appliquées avec succès aux œufs de *Petromyzon* (Weissenberg, 1929, 1933, 1934, 1936), de *Scyllium canicula* (Vandebroek, 1936), de la Truite (Pasteels, 1933, 1934, 1936*b*), de *Fundulus* (Oppenheimer, 1936). L'extension des conclusions de Vogt aux autres Anamniotes ne rencontrait aucune difficulté. Par ailleurs, une étude nouvelle de la gastrulation de l'Amphioxus, due à Conklin (1932), par la délicate analyse du "cell-lineage", permet d'envisager cette gastrulation typique sous un jour nouveau, de montrer son identité avec celle des Ascidies (Conklin, 1905) et de la comparer efficacement avec celle des Vertébrés. Mais c'est chez les Amniotes surtout que l'effort d'investigation devait être poursuivi. Chez les Oiseaux, les contradictions entre les données de Gräper et de Wetzel rendaient une nouvelle étude nécessaire (Pasteels, 1937*b*). Avant cela, toutefois, la gastrulation des Reptiles, qui apparaissaient comme un véritable rébus, devait être déchiffrée (Pasteels, 1937*a*).

Ces documents nouveaux nous permettent enfin d'envisager la gastrulation des Chordés sous un jour unitaire (cf. Pasteels, 1937*b*; Dalcq, 1937, 1938; Vandel, 1937). Sans doute, des lacunes persistent encore. Certains groupes zoologiques tels que les Myxines, les Ganoïdes, les Dipneustes, les Gymnophiones, les Crocodiliens, et surtout les Mammifères mériteraient d'être étudiés à leur tour. Mais ce que l'on en sait déjà permet bien d'affirmer qu'ils ne nous réservent aucune surprise. Plus délicate est la question de la mise en place de l'entoblaste et de la signification du stade didermique chez les Amniotes. Nous nous y arrêterons plus loin.

Il serait évidemment désirable de pouvoir envisager une vue comparative de la gastrulation chez tous les Métazoaires. Malheureusement, les études ne sont pas encore suffisamment poussées chez l'ensemble des Invertébrés pour que cette comparaison puisse être faite de façon utile, dès maintenant. On ne dispose en effet de documents sûrs que chez l'Oursin; aussi chez les Annélides et Mollusques qui ont été analysés pendant la première décade de ce siècle par l'admirable méthode du "cell-lineage" de l'école américaine (E. B. Wilson, E. G. Conklin, F. R. Lillie).

Le cas de l'Oursin est assez significatif: c'est un objet surabondamment travaillé et chez lequel la gastrulation apparaît d'une simplicité élémentaire. Il a fallu cependant que Hörstadius (1935) utilise la coloration vitale au bleu de Nil pour redresser une erreur concernant la limite d'invagination. Il n'est pas excessif d'affirmer que tous les documents anciens (sauf ceux du "cell-lineage" qui sont impeccables) sont à revoir. Certains mériteraient, en raison même de leur gros intérêt théorique, une étude particulièrement attentive, par exemple, cette affirma-

tion de Lignau en 1911 qui, chez le Diplopode *Polydesmus*, fait s'invaginer entoblaste et mésoblaste par des blastopores différents (cf. Dawydoff, 1928, p. 538).

Nous devrons donc, quoique à regret, nous limiter aux Chordés. Nous adopterons la nomenclature des feuillets proposée par Dalcq & Gérard dans la 2ème édition du *Traité d'Embryologie* de A. Brachet (1935).

## II. REVUE DE LA GASTRULATION CHEZ LES DIFFÉRENTS CHORDÉS

### 1. AMPHIBIENS

Le mémoire de Vogt (1929*a*), consacré à l'étude approfondie de la gastrulation des Urodèles et des Anoures, est venu renouveler intégralement nos notions sur la gastrulation des Vertébrés. Il convient donc d'envisager les résultats de Vogt en détail, quitte à y ajouter quelques précisions nouvelles acquises depuis lors. L'auteur a associé les deux méthodes: sa technique des marques colorées et l'étude microscopique usuelle.

Les marques colorées sont réalisées par le contact d'un fragment d'agar imprégné préalablement de bleu de Nil, de rouge neutre ou de brun Bismarck. La coloration qui se fixe sur les plaquettes vitellines et surtout sur le pigment n'a aucune tendance à la diffusion ultérieure. Sauf surcoloration éventuelle, facile à éviter, la vitalité des cellules tatouées ne subit aucune altération. On a depuis réussi à conserver soit le bleu de Nil (Lehmann, 1928), soit le brun Bismarck (Weissenberg, 1929) sur coupes fixées, mais les résultats de Vogt ont été acquis indépendamment de ces perfectionnements.

Les marques (de préférence de coloration alternée) sont repérées par rapport à l'axe ovulaire et au plan de symétrie bilatérale. Des dessins à la chambre claire, des dissections lorsque les cellules colorées se sont enfoncées au sein du germe indiqueront le chemin qu'elles ont parcouru et leur destinée finale dans le jeune embryon muni de ses organes primordiaux. Un repérage systématique de tous les points de la surface de la blastula constitue ainsi le point crucial de l'étude de la gastrulation. Une telle étude aboutit à trois ordres de résultats que nous envisagerons successivement. (1) Les différents organes primordiaux de la jeune larve dérivent de portions définies du germe prégastruléen. Il existe donc dans la blastula, pour chacun de ces organes un groupe cellulaire qui en constitue la source matérielle. On peut donc délimiter sur la blastula une véritable carte géographique de ces *ébauches présomptives*. Ce serait toutefois une grosse erreur que de conférer à cette notion d'ordre purement topographique une nuance de causalité, que de les confondre par exemple avec des "localisations germinales". Ce dernier terme impliquerait en effet que le territoire considéré possèderait les causes intrinsèques de sa destinée. Or pour les ébauches présomptives, rien n'est moins vrai. S'il existe, par exemple, un croissant dorsal de cellules blastuléennes dont les dérivés constitueront le système nerveux, on sait par ailleurs que ces propriétés neurales leur sont conférées au contact du chordomésoblaste sous-jacent, du centre organisateur de Spemann. (2) Les territoires de la blastula, pour parvenir à leur emplacement définitif dans la jeune larve, suivent des chemins bien définis. L'ensemble de ces

déplacements des cellules, dont l'amplitude était insoupçonnée, déterminera les changements successifs et si caractéristiques de la forme gastruléenne. Ces mouvements de manœuvre de la foule cellulaire peuvent être analysés en grandes composantes que Vogt dénomma "*Gestaltungsbewegungen*", les ***mouvements morphogénétiques***. (3) Ces faits nouveaux permettent enfin une ***interprétation*** nouvelle de la gastrulation.

Avant d'entrer dans le détail de ces trois ordres de résultats, il convient de noter que des marques colorées ont été appliquées dès le stade morula sur l'œuf de Triton (Vogt, 1929*a*); et de Grenouille (Votquenne, 1934), ce qui a pu démontrer l'existence de mouvements prégastruléens, liés au creusement du blastocœle (Vintemberger, 1933, 1934) et consistant en un tassement général des ébauches vers le pôle inférieur de l'œuf. Sur l'œuf insegmenté, en l'absence de cellularisation, l'application de la méthode est peu fructueuse, les particules colorées étant déplacées et séparées par les remaniements intraovulaires considérables, encore que mal connus. A ce stade, le marquage électrolytique de Ancel & Vintemberger (1934) est d'un meilleur rendement et a pu élucider le remaniement cortical aboutissant à la formation du croissant gris des Anoures.

### (*a*) *Le plan des ébauches de la blastula*

Ces plans tels qu'ils résultent des recherches de Vogt sont représentés sur la fig. 1 *a* (Triton) et *b* (Bombinator). L'œuf y est vu de profil, son pôle inférieur en bas, son côté dorsal à droite, son côté ventral à gauche. On sait que de coutume, les œufs des Amphibiens présentent successivement, de haut en bas, trois zones distinctes: une région animale à petites cellules pigmentées, une zone équatoriale "marginale" à cellules grisâtres, enfin un amas inférieur de cellules fortement chargées de vitellus et de ce fait très grosses et blanches. La zone marginale constitue une ceinture, plus large du côté dorsal, comprise entre les deux courbes *bl.* (blastopore) et *l.i.* (limite d'invagination). Tant chez les Urodèles que chez les Anoures, on y trouve dorsalement tout juste au-dessus du blastopore, l'entoblaste du pharynx; immédiatement au-dessus le matériel, mal délimité, de la plaque préchordale; plus haut encore, la chorde dorsale (ponctuée). Celle-ci se prolonge en deux cornes latérales dans la zone marginale latérale.

Cette zone latérale diffère chez les Urodèles et les Anoures. Chez les premiers en effet le tracé du blastopore suit exactement la limite ento-mésoblastique tandis que chez les seconds, il existe dans la partie basse de la zone marginale une languette d'entoblaste correspondant à la voûte du tube digestif. Chez les Anoures, cet entoblaste va cacher complètement les lames latérales qui sont partiellement visibles en surface chez les Urodèles. Chez tous les Amphibiens, les somites antérieurs peuvent être délimités sous forme de bandelettes obliques allant en haut se raccorder aux prolongements latéraux du territoire chordal. Vogt n'a pas exploré les zones ventro-latérales. Elles sont laissées en blanc chez les Anoures; sur le plan des Urodèles nous voyons un territoire tronco-caudal dans la partie ventrale de la ceinture marginale, se prolongeant en une mince banguette au-dessus de la chorde.

Des recherches toutes récentes sont venues nous apporter des précisions supplé-

mentaires. Reprenant l'étude de la fonction de la queue, O. Nakamura (1938) confirme chez *Triturus pyrrhogaster* (Boie) les données acquises par Bijtel (1931) chez l'Axolotl (cf. plus loin). De plus, l'auteur japonais délimite le territoire somitique sous forme d'ailes ne dépassant que de peu le plan médio-frontal, mais remontant ensuite au-dessus de la chorde, à la limite du territoire d'invagination, et même au-dessus de celle-ci, en ce qui concerne les somites caudaux (cf. fig. 4*a*).

Selon Nakamura, la zone marginale ventrale et ventro-latérale est constituée par du matériel allant former uniquement les lames latérales et non en partie le bourgeon caudal, comme le croyait Vogt. Toutefois Nakamura ne délimite pas les différents somites et ne donne qu'une indication générale sur la situation des premiers somites du tronc, des somites postérieurs, et des somites caudaux. Il paraissait cependant intéressant de voir comment ils se raccordent à la chorde et au matériel latéral; aussi ai-je été amené à rechercher ces précisions chez l'Axolotl. La fig. 2*a*, *b* nous offre

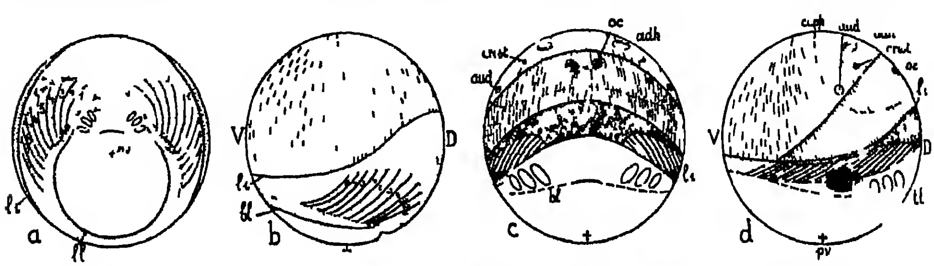


Fig. 1. Plan des ébauches de la jeune gastrula des Amphibiens (d'après Vogt, 1929*a*). (*a*) Triton vue inférieure. *l.i.* limite d'invagination; *bl.* lieu d'apparition du blastopore; *p v.* pôle végétatif; +, pôle inférieur de la jeune gastrula; ponctuation dense et foncée: champ chordal; ponctuation d'intensité moyenne: mésoblaste; les somites sont indiqués par des traits obliques et des chiffres; le matériel caudal par une ponctuation très espacée; aire hachurée. pronéphros; tirets denses. champ neural; tirets espacés · épiblaste; en blanc: l'entoblaste; ovales: poches branchiales. (*b*) Triton, vue latérale. Mêmes symboles. *D.* côté dorsal; *V.* côté ventral. (*c*) Bombinator, vue dorsale. Mêmes symboles *aud.* vésicules auditives; *crist.* cristallin; *oc.* chiasma et vésicules optiques; *adh.* glandes adhésives. (*d*) Bombinator, vue latérale. Mêmes symboles. *céph.* limite céphalique de l'ectoblaste.

à cet égard une nouvelle étape, qui n'est pas définitive non plus, car des recherches ultérieures devraient encore préciser certains points difficiles, tels que les limites exactes du champ chordal. Les contours douteux sont d'ailleurs marqués en tirets. En 2*a*, *b* nous voyons la blastula d'Axolotl respectivement de profil et de face. J'ai pu confirmer les données de Nakamura en montrant de plus que les somites constituent des bandes très inclinées, se rapprochant davantage de l'horizontale que Vogt ne les a représentés chez le Triton. Les dix premiers se trouvent dans la concavité de l'ébauche chordale. Celle-ci, comme Motomura (1932) l'a déjà montré chez *Hynobius*, est beaucoup plus ramassée que Vogt ne la figurait; ses limites latérales restent indécises toutefois. Les somites troncaux postérieurs sont représentés par de minces bandes, très serrées, mais très longues puisqu'elles s'étendent depuis les environs de la ligne médiane, au-dessus de la chorde jusqu'au delà du plan médio-frontal. Les somites caudaux présentent la même disposition, mais plus serrés encore. A l'exemple de Nakamura, je les figure au-dessus de la limite d'invagination, puisqu'ils passent dans la plaque et le tube médullaire avant leur mise en place

définitive. Le pronéphros que l'on peut colorer par des marques atteignant le blastopore à 90° du plan sagittal, se trouve dans le prolongement des 3ème et 4ème somites. Le canal de Wolff suit exactement la limite des somites et des lames

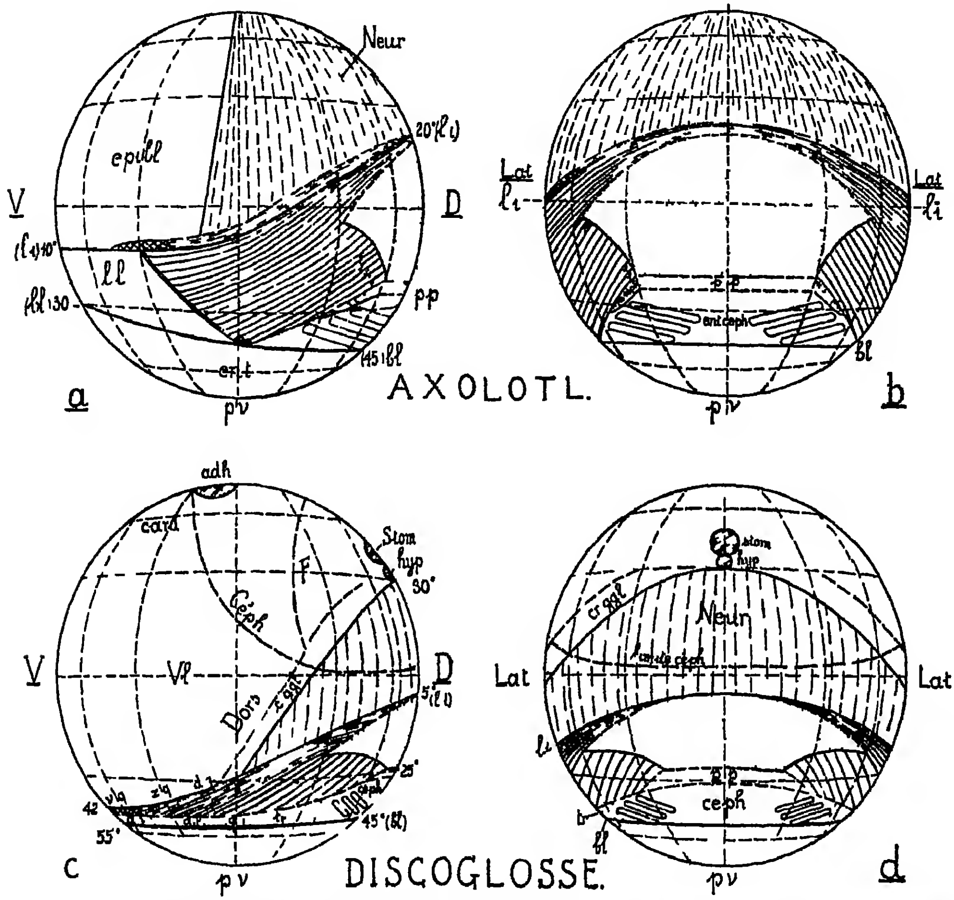


Fig. 2. Plan des ébauches de la blastula avancee de l'Axolotl et du Discoglosse. Indications générales: sphère vue en perspective; les latitudes et longitudes de 30° en 30° dessinés en tirets. Les limites bien précisées sont dessinées en traits pleins, les contours incertains en tirets. Aire en tirets (Neur.): neurectoblaste; aire ponctuée: chorde; stries obliques: somites; traits entrecroisés: mésenchyme de la nageoire ventrale de la queue, *D* côté dorsal; *V.* côté ventral; *p v.* pôle végétatif, *lat.* partie latérale; *p.p.* plaque préchordale; *l.i.* limite d'invagination; *bl.* lieu d'apparition du blastopore. (*a*) Axolotl, vue latérale. *Epibl.* épiblaste, *l.l.* lames latérales; *m c.* mésoblaste céphalique. (*b*) Axolotl, vue de face. *Mes.ceph.* mésoblaste céphalique; *ent.céph.* entoblaste céphalique. (*c*) Discoglosse, vue latérale. Indications dans l'ectoblaste; *card.* région cardiaque; *adh.* glandes adhésives; *céph.* limite céphalique; *F* limite faciale; *stom* stomodœum; *hyp.* hypophyse; *Vl.* flanc; *Dors.* dos; *c ggl.* crête ganglionnaire; *v.q.*, *a.q.*, *d.q*, portions ventrale, apicale et dorsale de la queue. Indications dans l'entoblaste: *céph.* tête; *tr* tronc; *q.* queue; *a.l.* parois latérales de l'anus; *a.v.* parois ventrales de l'anus. (*d*) Discoglosse, vue de face. Mêmes symboles.

latérales. Des recherches récentes d'O'Connor (1938) montrent toutefois que l'ébauche du canal de Wolff ne dépasse pas le 7ème segment. Quant aux lames latérales, il n'y a guère que leur partie postérieure qui puisse être entièrement colorable par des marques superficielles; pour les segments antérieurs, la partie ventrale du seg-

ment disparaît en profondeur et celà d'autant plus qu'il s'agit d'un segment situé plus en avant dans l'embryon; entre le 3ème et le 12ème somite, il n'y a que la partie dorsale des lames latérales qui soit superficielle; les lames entières des trois premiers segments sont profondes, et même, comme l'avait déjà vu Vogt, une partie des deux premiers somites.

J'ai fait un repérage analogue sur l'œuf de Discoglosse, dont le plan avait été publié (Pasteels, 1936*a*) sans le tracé des somites individuels. Sur la fig. 2*c*, *d*, nous voyons que ce plan présente la même allure générale que chez l'Axolotl, mais la zone marginale est plus aplatie, et la chorde et les somites sont rejetés davantage vers le côté ventral. La forme générale du territoire somitique est la même; mais si les somites postérieurs n'empiètent que peu au-dessus de la chorde en revanche ils occupent toute la zone ventro-latérale. Les somites individuels sont moins inclinés. L'ensemble des lames latérales ainsi que le pronéphros et le canal de Wolff sont cachés en profondeur, vraisemblablement sous la crête entoblastique sus-blastoporale si caractéristique des Anoures. Celle-ci a pu être subdivisée en territoires céphalique (*t.*), troncal (*tr.*), caudal (*q.*, constituant tout l'entoblaste caudal), anal latéral (*l.a.*), anal ventral (*v.a.*). Il est curieux de constater la place considérable que prend l'anus sur la surface de l'œuf.

Quant à l'ectoblaste, on sait depuis Vogt que, chez les Urodèles (figs. 1*a*; 2*b*) il est à peu près subdivisé en deux parties égales, une dorsale constituant le neurectoblaste,[1] une ventrale l'épiblaste. Chez les Anoures, où le chordo-mésoblaste est beaucoup plus tassé, le neurectoblaste l'est tout autant et forme un croissant dorsal situé aux environs de l'équateur (fig. 2*c*) ou au-dessus de lui (fig. 1*b*). Cette dernière figure montre en pointillé la délimitation de l'ectoblaste de la tête, le lieu de chiasma optique, de la vésicule auditive (Vogt). Chez le Discoglosse (Pasteels) on pourra voir (fig. 2*c*, *d*) la topographie des crêtes ganglionnaires (*c.g.*), de l'épiblaste hypophysaire (*h*), stomodaeal (*st*), de l'ensemble de la face (*F*), de l'épiblaste de la tête, des glandes adhésives de la larve (*adh.*), de la région cardiaque (*card.*), des branchies (*br.*), du ventre (*V*), des flancs (*V.l.*), des parties ventrale (*v.q.*), apicale (*z.q.*) et dorsale (*d.q.*) de la queue.

Pour être complet, le repérage devrait être poursuivi en profondeur. A défaut de marques colorées, il faut se contenter de données expérimentales. A ce point de vue, les meilleures sont les explantations de parties de germe cultivées en solution physiologique telles que les a réalisées Holtfreter (1938 *a*, *b*). Toutefois, ces données mêmes ne peuvent être considérées qu'avec circonspection, car les conditions d'explantation peuvent modifier l'évolution des territoires. En effet, si nous comparons les résultats de Holtfreter avec le plan des ébauches, nous voyons en premier lieu que l'ensemble du chordo-mésoblaste peut, après explantation, donner indifféremment de la chorde, des somites, du système nerveux et de l'épiblaste. En revanche, Holtfreter (1938*a*, p. 629) croit que pour des différentiations telles que le rein, le cœur, le sang, les divers organes entoblastiques, de telles métaplasies sont plus rares et que ses données peuvent dans ce cas fournir des renseignements sur la localisation

[1] Le terme de neurectoblaste a été proposé de façon heureuse par Celestino da Costa (1938) pour remplacer le terme de neuroblaste, prêtant à confusion de Dalcq & Gerard.

des ébauches. Ceci n'est pas certain. En effet, Holtfreter fait dériver le pronéphros des Urodèles de la zone marginale interne, le maximum de tubes néphrétiques se trouvant dans des explantats provenant de la zone située à 35 à 40° du plan sagittal. Or, nous avons vu que pronéphros et canal de Wolff chez l'Axolotl proviennent de matériaux superficiels, le *pronéphros se trouvant à 90° du plan sagittal*, le canal de Wolff à la limite des somites et des lames latérales. Ici aussi, nous trouvons une discordance marquée entre les données d'explantation et d'observation. Acceptons donc sous toutes réserves, en attendant confirmation ultérieure, que chez les Urodèles le matériel cardiaque se trouverait dans la zone marginale interne à environ 35° du plan sagittal, le sang s'y situerait dans la région ventrale (Holtfreter, 1938*a*, p. 629). Les matériaux entoblastiques se trouveraient au plancher de la cavité de segmentation: foie encadré de l'estomac dorsalement, intestin ventralement; la région du pôle végétatif étant constituée de vitellus nutritif sans signification organogénétique. Une disposition analogue se retrouverait chez les Anoures (Holtfreter, 1938*b*, p. 721). Notons qu'ici, le pronéphros est certainement interne et très probablement, vu la disposition des somites, plus médian que chez les Urodèles.

L'essentiel du plan des ébauches des Amphibiens peut être *résumé* par les trois principes suivants: (*a*) l'œuf peut être de haut en bas divisé en trois zones: une zone animale, ectoblastique, non invaginée, une zone marginale moyenne, invaginée au-dessus du blastopore, enfin une calotte vitelline inférieure qui s'invaginera sous le blastopore. (*b*) Les organes axiaux de l'embryon futur (système nerveux; chorde, somites) sont disposés en croissants transversaux. (*c*) Les territoires médiodorsaux de l'embryon se trouvent déjà sur la ligne médiodorsale de la blastula.

### (*b*) *Les mouvements morphogénétiques*

Ces mouvements ont été systématisés par Vogt en grandes composantes qui sont: l'*invagination*, la *convergence dorsale*, la *divergence ventrale* et l'*épibolie*.

(1) *L'invagination* concerne à la fois la zone marginale (chordomésoblaste seul chez les Urodèles, plus la crête entoblastique chez les Anoures) et la masse vitelline entoblastique. Le sillon blastoporal apparaît à la limite de la zone marginale et du vitellus, en premier lieu du côté dorsal, puis se continue progressivement par deux lèvres latérales ("blastopore en fer à cheval"), puis par une lèvre ventrale ("blastopore annulaire") en suivant le tracé de la courbe *bl.* des figs. 1 et 2. L'amas de cellules circonscrit par ce blastopore annulaire (cf. fig. 4*c*) se dénomme "bouchon vitellin" (vu en coupe sagittale sur la fig. 3*b*, *e*). Mais l'orifice blastoporal se rétrécit de plus en plus et le bouchon vitellin se réduit pour être englouti à l'intérieur de l'œuf. Au moment de l'apparition de la plaque médullaire, ce blastopore n'est plus qu'une fente qui devient l'anus chez les Urodèles tandis que chez les Anoures il se subdivise en anus et canal neurentérique. La zone marginale s'invaginant au-dessus du blastopore, le bouchon vitellin en dessous, c'est entre les deux qu'il faudra rechercher la lumière archentérique.

Avant d'entrer dans le détail, remarquons l'étendue de ce mouvement d'invagi-

nation. C'est presque toute la moitié inférieure de la surface blastuléenne qui se replie à l'intérieur de l'œuf. Or, avant le mémoire de Vogt, on était loin d'être unanime à attribuer la formation des feuillets profonds à des déplacements si étendus des cellules. On considérait plus volontiers qu'il se passait des regroupements limités de cellules se trouvant déjà en profondeur ("clivage gastruléen" de A. Brachet) et surtout de la "prolifération" au niveau du blastopore.

Le clivage gastruléen joue un rôle réel mais accessoire, et chez les Anoures seulement, au début de la gastrulation, au niveau de la zone marginale interne, sur le

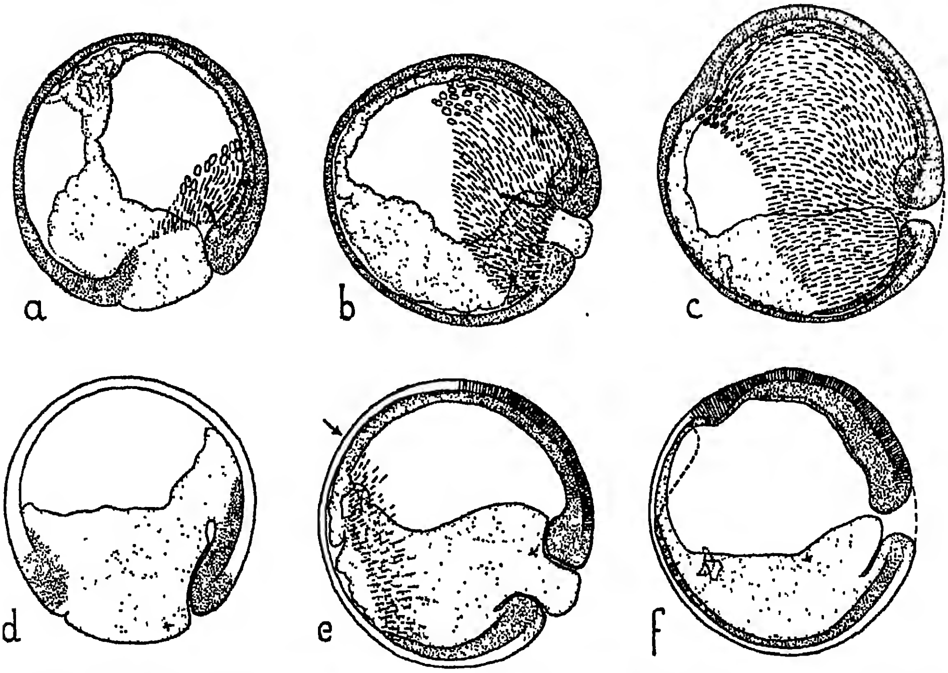


**Fig. 3. Reconstructions de coupes sagittales: *a, b, c,* Urodèles; *d, e, f,* Anoures (d'après Vogt, 1929, les flèches des figs. *a* et *b* ont été ajoutées). Les tirets indiquent la progression du matériel mésoblastique.**

pourtour du plancher blastocœlien, les cellules vitellines profondes se détachent de la couche superficielle et servent ainsi d'avant-garde aux cellules invaginées.

Quant à la prolifération, il a suffi trop souvent d'apercevoir quelques mitoses au hasard des coupes pour la mettre en cause. En réalité, une statistique rigoureuse n'a jamais été faite et la démonstration qu'il existe réellement une *croissance*, condition première pour que la "prolifération" puisse provoquer une déformation du germe, n'a jamais été envisagée. Pour le Discoglosse, Wintrebert (1935) parle volontiers d'un "centre initiateur mitogénétique", mais sans aucune base objective (Pasteels, 1936*a*). Récemment Bragg (1938), dénombrant dans les divers territoires de l'œuf la proportion des mitoses par rapport au nombre total des cellules chez l'Anoure *Bufo cognatus*, en arrive à des conclusions assez singulières qui devraient faire admettre que le développement de ce crapaud serait totalement différent de

tout autre Amphibien. En réalité, la méthode de Bragg, comme celle de toute l'école de Richards (1935, etc.) n'est exacte qu'en apparence, mais entachée d'une erreur de statistique et d'une erreur de raisonnement. Nous y reviendrons plus loin.

Chez tous les Amphibiens la première lèvre blastoporale apparaît en un point de l'entoblaste qui correspond au récessus hépatique de la jeune neurula. C'est donc tout l'entoblaste céphalique qui franchira en premier lieu la lèvre dorsale du blasto-

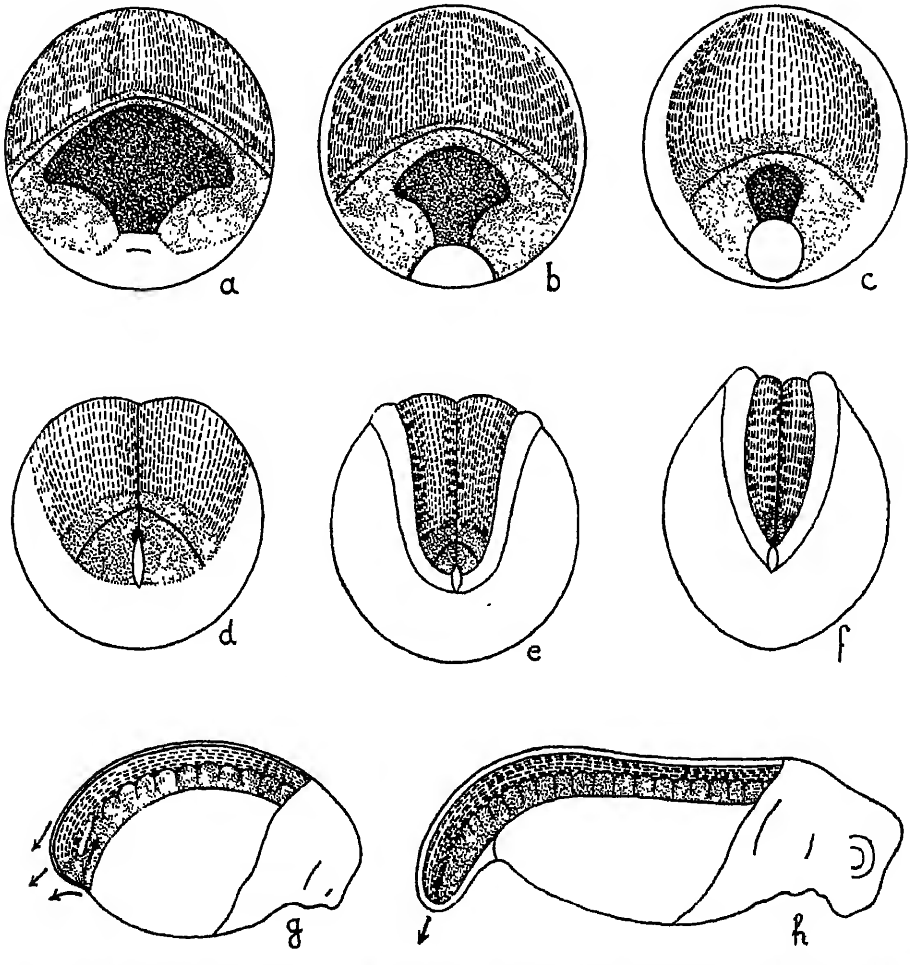


Fig. 4. Etapes de la gastrulation et de la formation de la queue chez *Triturus pyrrhogaster* (d'après Nakamura, 1938, légèrement modifié). Ponctuation dense: chorde; ponctuation claire: somites; tirets: neurectoblaste.

pore pour constituer l'extrémité antérieure de l'archentéron. Il est curieux de constater combien ce matériel qui occupe peu de place à la surface de la blastula (cf. figs. 1 et 2) va s'étirer pour constituer cette longue lame cellulaire allant du récessus hépatique jusqu'au niveau de l'extrémité antérieure du système nerveux. Des recherches au cours desquelles des marques faites chez le Discoglosse ont pu être retrouvées sur coupes microscopiques, ont montré qu'une grosse part de ce

matériel entoblastique de la tête doit provenir de la profondeur, de la zone avoisinant la partie dorsale du plancher du blastocœle (Pasteels, 1936*a*). L'invagination suivant son cours, ce sera bientôt la "plaque préchordale" (matériel complexe comprenant de l'entoblaste dorsal et le mésoblaste médian de la tête) qui passera à son tour autour de la charnière blastoporale. Pendant ce temps, en dessous de la lèvre blastoporale, au niveau de son plancher, les cellules vitellines sont elles aussi attirées en avant en effectuant un mouvement de bascule, de façon que leurs surfaces libres viennent constituer le plancher de l'archentéron. Nous en sommes à présent à un stade représenté sur la fig. 4*b*. L'extrémité antérieure de l'archentéron est constituée; elle constitue un cul-de-sac continu de toute part (cf. fig. 3*a*, *d* qui représentent un stade un peu plus avancé).

Lorsque l'entoblaste céphalique s'est ainsi invaginé, l'ébauche chordale atteint à son tour la lèvre dorsale du blastopore (fig. 4*b*). A ce moment, la lèvre blastoporale s'est étendue sur les côtés (blastopore en faucille). Le mode d'invagination est dès ce moment un peu différent chez les Urodèles et chez les Anoures. Chez les premiers, nous avons vu que le sillon blastoporal suit, latéralement, en dehors du matériel céphalique, exactement la limite ento-mésoblastique (cf. figs. 1 *a* et 2 *a*, *b*). Dès le début de l'invagination, il se passe à ce niveau *une véritable rupture entre entoblaste et mésoblaste*. Les deux matériaux, séparés par une véritable coupure vont suivre des chemins différents. L'entoblaste subit un mouvement de bascule d'avant en arrière de telle manière que sa face primitivement superficielle vienne constituer le plancher de l'archentéron. En même temps, au niveau du blastopore, les deux lèvres qui se sont séparées du mésoblaste se redressent et vont ainsi par un mouvement progressif constituer les deux crêtes que l'on voit sur les côtés de l'archentéron (fig. 5). Nous voyons que cette dernière cavité, qui était close de toutes parts en avant, est donc interrompue en arrière. La fig. 5 nous montre clairement que l'on passe du plancher vers les faces latérales sans interruption, mais qu'entre ces crêtes entoblastiques latérales et la voûte archentérique constituée par la chorde existe une solution de continuité. En effet, le matériel chordal, sur la ligne médiane a suivi le préchordal pour se placer donc à la voûte archentérique. Latéralement la plaque chordale se continue par un "manteau mésoblastique" qui s'est invaginé en même temps qu'elle, mais au niveau des lèvres blastoporales latérales. Chose caractéristique, *ce mésoblaste n'est pas en rapport avec l'archentéron et ne l'a jamais été*. Les bords du mésoblaste après s'être séparés de l'entoblaste franchissent le blastopore, mais au-dessus de lui. Ce mouvement se fait avec un certain retard par rapport à l'invagination archentérique. Celà se voit clairement sur les reconstructions de la fig. 3 *a*, *b*, *c* où l'on voit d'une part l'étendue de l'archentéron au stade de blastopore annulaire (*a*), de bouchon vitellin (*b*), de blastopore en fente (*c*). On y comprendra d'autre part la progression du manteau mésoblastique qui s'effectue avec un retard considérable par rapport à l'invagination archentérique. Tenant compte de ce fait et du mode de constitution des parties latérales de l'archentéron par les crêtes entoblastique, il est clair que le mésoblaste, après avoir franchi le blastopore, va cheminer *entre l'entoblaste et l'ectoblaste* (cf. fig. 5). Pour bien apprécier son trajet, il faut tenir compte de sa topographie et de la chronologie de son

déplacement. Les cellules mésoblastiques qui se trouvent au contact du blastopore, au niveau de la rupture avec l'entoblaste, s'invagineront bien entendu avant celles qui se trouvent au-dessus d'elles; mais ce seront les éléments de la zone marginale profonde qui s'invagineront en tout premier lieu. Ils se trouvent aux avant-postes du manteau mésoblastique. Par ailleurs, l'invagination, qui commence dorsalement, se poursuit, progressivement sur les côtés pour n'apparaître qu'en dernier lieu du côté ventral. Ceci dit, si nous retournons à la fig. 3*a*, nous y voyons un manteau mésoblastique de forme triangulaire. Dorsalement et en avant, le matériel figuré par de petits ovales figure le mésoblaste préchordal en connexion plus ou moins étroite avec la voûte archentérique; en arrière de lui se trouveront les premières lames latérales; plus en dehors le matériel qui vient de s'invaginer et qui provient de la zone marginale interne, ira constituer le cœur. A un stade ultérieur (fig. 3*b*), les somites antérieurs se sont invaginés près de la ligne médiane, en même temps que les lames latérales moyennes qui ont fait suite au matériel cardiaque, tandis que ventralement l'invagination en est à son début et concerne le matériel interne (sang).

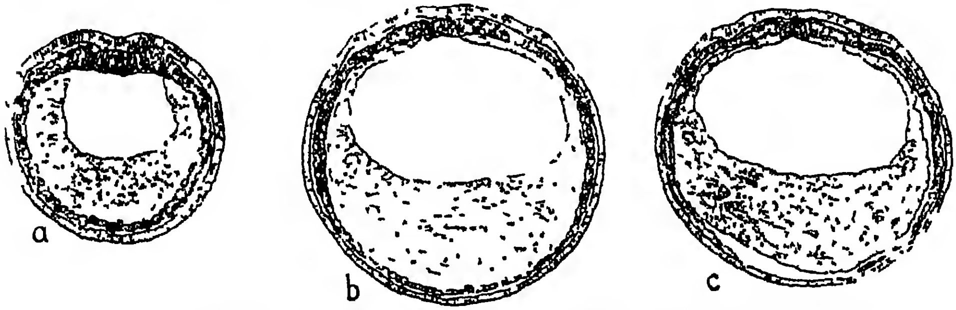


Fig. 5. Trois coupes sagittales passant par trois niveaux d'une jeune neurula de Pleurodèles (d'après Vogt, 1929*a*).

Chez les Anoures le principe général est le même, à une différence près: la séparation entre ento- et mésoblaste est tardive. Nous savons en effet que le sillon blastoporal ne sépare pas ces deux ébauches mais qu'il apparaît au sein même de l'entoblaste entre ce qui va constituer les crêtes latérales de l'archentéron et son plancher. Ces crêtes qui cachent le matériel des lames latérales vont s'invaginer en même temps que celles-ci dans un mouvement commun. La séparation entre ento- et mésoblaste ne se fera qu'à l'intérieur du germe. Il en résultera qu'on ne retrouvera pas ici la dissociation chronologique entre la formation de l'archentéron et l'invagination du manteau mésoblastique. Comme on peut le voir sur la fig. 3 *d*, *e*, *f* (à comparer avec *a*, *b*, *c*) chez les Anoures les deux vont de pair et l'invagination du mésoblaste est donc plus rapide et plus précoce que chez les Urodèles. Vogt a d'ailleurs noté chez ces derniers qu'au moment de l'apparition de la plaque médullaire il n'y a qu'une dizaine de somites qui soient invaginés et par conséquent que toute la moitié postérieure du tronc et toute la queue doivent encore s'enfoncer en profondeur par le petit pertuis blastoporal; en revanche, chez les Anoures, tous les segments troncaux et même une partie des éléments caudaux (recherches personnelles inédites) sont à ce moment déjà en place.

Vogt a constaté que les somites caudaux des Anoures sont invaginés au moment où le blastopore linéaire se soude en sa partie médiane pour se subdiviser en canal neurentérique et anus. C'est au moment de cette soudure et par celle-ci que le matériel caudal s'enfonce en profondeur. Ces données sont à présent confirmées et complétées par des recherches personnelles qui seront publiées sous peu.

Chez les Urodèles, Vogt croyait aussi, à l'encontre de Bijtel & Woerdeman (1928), que les somites troncaux postérieurs et caudaux s'invaginaient par les lèvres latérales de la fente blastoporale, en arrière de la plaque médullaire. Des travaux ultérieurs de Bijtel (1931, 1936) chez l'Axolotl, de Nakamura chez *Triturus pyrrhogaster* sont venus donner raison aux auteurs néerlandais.[1] Nous avons vu que les somites postérieurs constituent sur la blastula de l'Axolotl (cf. fig. 2 *a*, *b*) de longues bandes obliques occupant toute la partie haute de la zone marginale, au-dessus et en dehors de la chorde. Ce matériel haut qui sera invaginé en dernier lieu, se retrouve, après convergence vers la ligne médiane (cf. plus loin), ramassé dans la partie postérieure de la plaque médullaire naissante (fig. 4*d*). On y voit le bout de la chorde sur le point de s'enfoncer (pointillé dense) et une zone mésoblastique (pointillé mince) divisée elle-même en deux régions par une ligne courbe. Celle-ci n'est que la limite d'invagination des figs. 1, 2, et que l'on retrouve en la fig. 4*a*. Tout ce qui est en dessous de cette limite est troncal et s'invagine par la partie antérieure du blastopore (fig. 4*e*). Quant au mésoblaste caudal, il reste compris dans la plaque médullaire (fig. 4*f*) et passe même dans le tube médullaire (fig. 4*g*). Mais à ce moment l'extension de la chorde vient replier la partie postérieure du tube en deux: une partie dorsale nerveuse, et une partie ventrale venant constituer les somites caudaux (fig. 4 *g*, *h*). Il est vraiment curieux de constater que ce territoire somitique caudal situé sur la blastula à la limite de la zone marginale et du neurectoblaste semble littéralement hésiter entre ces deux destinées et possède une véritable dynamique de transition.

Chez l'Anoure Discoglosse, Wintrebert (1935) avait cru montrer que l'allongement du bourgeon caudal serait dû à un enfoncement transversal de matériaux à travers la plaque médullaire vers la voûte archentérique sous-jacente (soi-disant: "ligne primitive"). Des observations restées inédites, faites au Laboratoire d'Embryologie de Bruxelles, que j'ai pû contrôler et suivre de très près, n'ont pu vérifier ces conclusions de Wintrebert.

(2) *Convergence–Extension*. Ces deux mouvements peuvent difficilement être dissociés l'un de l'autre. Nous avons vu en effet que les organes axiaux sont disposés en croissants transversaux à la surface de la blastula. Pour constituer l'axe embryonnaire ils devront subir un mouvement complexe d'étirement antéro-postérieur (extension) et de tassement latéral (convergence). Le fait est bien visible pour le croissant neurectoblastique, qui reste en surface (stades successifs de la fig. 4); nous comprendrons facilement comment ce croissant évolue progressivement en la raquette qui est constituée par la plaque médullaire. Goerttler (1925) a décrit pour la première fois cette évolution complexe sous le terme militaire de "Schwenkung" (conversion). Cette combinaison de convergence–extension laisse subsister pendant toute l'évolution sur la ligne médiane du matériel qui s'y trouvait médian depuis le

[1] Cf. Addendum, p. 106.

début. Ce matériel s'étire au maximum. Nous avons là une différence radicale avec la concrescence de His qui supposait une *fusion* médiane de moitiés primitivement séparées.

Dans le matériel marginal, convergence et extension débutent en surface et se poursuivent en profondeur. C'est l'extension de la zone marginale ventrale qui va contribuer à amener du matériel vers le blastopore; tandis que la convergence va tasser la chorde vers la lèvre dorsale (voir les figs. successives de 4); elle va d'autre part amener les somites vers la lèvre latérale (cf. fig. 4). L'extension est une caractéristique des matériaux médians (chorde), la convergence est le propre des matériaux paramédians (somites). L'une et l'autre sont d'autant plus accentuées que les matériaux sont d'invagination plus tardive et plus postérieurs dans l'embryon (chorde postérieure, somites caudaux).

L'extension est surtout forte dans les phases tardives, post-gastruléennes. C'est à ce moment que la chorde vient faire saillie dans le bourgeon caudal (voir plus haut). On observe à ce stade un recul caractéristique de la chorde par rapport aux somites qui s'étaient invaginés en même temps qu'elle (Vogt). En profondeur, la convergence entraîne le matériel somitique vers les flancs de la chorde. La combinaison de l'invagination et de la convergence peut se traduire par les lignes spiroïdes des fig. 3 *a*, *b*. Il existe également une convergence tardive qui, par un mouvement transversal va littéralement tasser le dos et fermer l'hiatus entoblastique dorsal (fig. 5). Les crêtes entoblastiques se relèvent de plus en plus, viennent au contact de la chorde, semblant la déprimer en gouttière (fig. 5*c*). Des aspects tels que ceux de cette dernière figure ont donné lieu à l'interprétation abusive d'une origine entoblastique de la chorde. Plus tard, les somites se tasseront davantage, pousseront sous la chorde des prolongements qui constitueront l'hypochorde. La soudure des lèvres entoblastiques viendra enfin clore définitivement la cavité digestive. On trouvera le détail de cette évolution dans une note de Vogt (1929*b*) et surtout dans le mémoire de son élève Mayer (1931).

Le matériel des lames latérales et surtout du cœur et du sang qui constitue le bord antérieur du manteau mésoblastique est refoulé ventralement (fig. 3). C'est ce que Vogt appelle la divergence ventrale. Notons que cette divergence est tardive et que la partie supérieure des lames latérales ainsi que la pièce intermédiaire, subissent une certaine convergence.

(3) *Épibolie*. L'enveloppement progressif du germe par l'ectoblaste nécessite une forte augmentation de surface. Au niveau de l'épiblaste, cette augmentation de surface se fait par un mouvement non polarisé: une *épibolie* dans tous les sens. Il est à noter cependant qu'au sein du neurectoblaste, une certaine épibolie se combine à l'extension-convergence que ce matériel subit au contact du chordo-mésoblaste qui induit, "évoque" le système nerveux sus-jacent.

### (*c*) *Les conclusions nouvelles apportées par Vogt*

(1) La correspondance éventuelle des axes ovulaire et embryonnaire est une question en partie mal posée. Il existe bien dans la blastula un côté entièrement dorsal et un côté ventral et l'*axe dorso-ventral* de l'œuf correspond au même axe de

l'embryon. En revanche, un axe céphalo-caudal pour la blastula n'a aucun sens. Car pour définir "l'extrémité" caudale de la blastula, il faudrait par exemple sur la fig. 2 prendre des territoires aussi hétéroclites que la ligne supérieure de la chorde, inférieure du système nerveux, tout le dernier somite, l'ectoblaste caudal postérieur (*z.q.*), l'entoblaste caudal (*q.*).

(2) La *concrescence* ne joue aucun rôle dans la morphogénèse des Amphibiens, il n'y existe en aucune façon de soudure médiane de parties paires primitivement séparées. Les territoires médio-dorsaux de l'embryon étaient déjà médians dès la blastula.

(3) Tout aussi erronée est la *théorie cœlomique* de Hertwig. Le mésoblaste ne se développe pas de dedans en dehors à partir de la voûte archentérique, mais bien en direction opposée *de dehors en dedans vers cette voûte*. La soi-disant fente cœlomique de Hertwig ne sépare pas un élément splanchnique d'un élément viscéral mais bien l'ensemble du mésoblaste des crêtes entoblastiques. Le véritable cœlome est d'apparition plus tardive et ne se creuse que dans les lames latérales qui n'ont jamais eu aucun rapport avec l'archentéron.

(4) La *chorde* n'est pas d'origine entoblastique mais est issue du chordomésoblaste marginal.

(5) En ce qui concerne la *théorie de la gastraea*, Vogt est moins catégorique. Evidemment, la gastrula des Amphibiens, tridermique d'emblée ne satisfait pas le schéma didermique de Haeckel; la paroi archentérique n'est pas d'une venue, mais elle est complexe, composite, résultant d'une opposition secondaire d'éléments hétérogènes. Toutefois, Vogt propose de garder la notion de la gastraea didermique originelle avec, comme correctif, une zone blastoporale donnant naissance au chordo-mésoblaste. Ceci pour échapper à l'éventualité de faire dériver le feuillet moyen soit de l'entoblaste, soit de l'ectoblaste. Nous verrons plus loin ce qu'il faut en penser.

(6) La notion de *céphalo-* et *notogénèse*, les notions de *croissance appositionnelle*, de *centres de croissance* sont également réfutées. D'avant en arrière, dans tout l'embryon, les organes primordiaux sont mis en place par les mêmes mouvements morphogénétiques.

### 2. Téléostéens

L'étude de la gastrulation de l'œuf télolécithique des Téléostéens présentait un gros intérêt car c'est précisément à leur propos que His, et plus tard Kopsch, sous une autre forme (voir plus haut), ont voulu établir la notion de concrescence. On aurait pu croire, à priori, que des œufs fortement transformés par l'accumulation du vitellus auraient pu, peut-être, contrairement à ce qui se passe chez les Amphibiens, se développer par concrescence. Il n'en est rien. Le plan des ébauches et les mouvements morphogénétiques ont été analysés chez la Truite (Pasteels, 1936*b*; notes préliminaires en 1933, 1934) et chez *Fundulus* (Oppenheimer, 1936). La fig. 6, qui concerne la Truite, nous montrera aisément à quel point la similitude avec le plan et les mouvements des Amphibiens est frappante. Pour la compréhension de cette figure, notons qu'elle représente uniquement la partie embryonnaire, et vue de

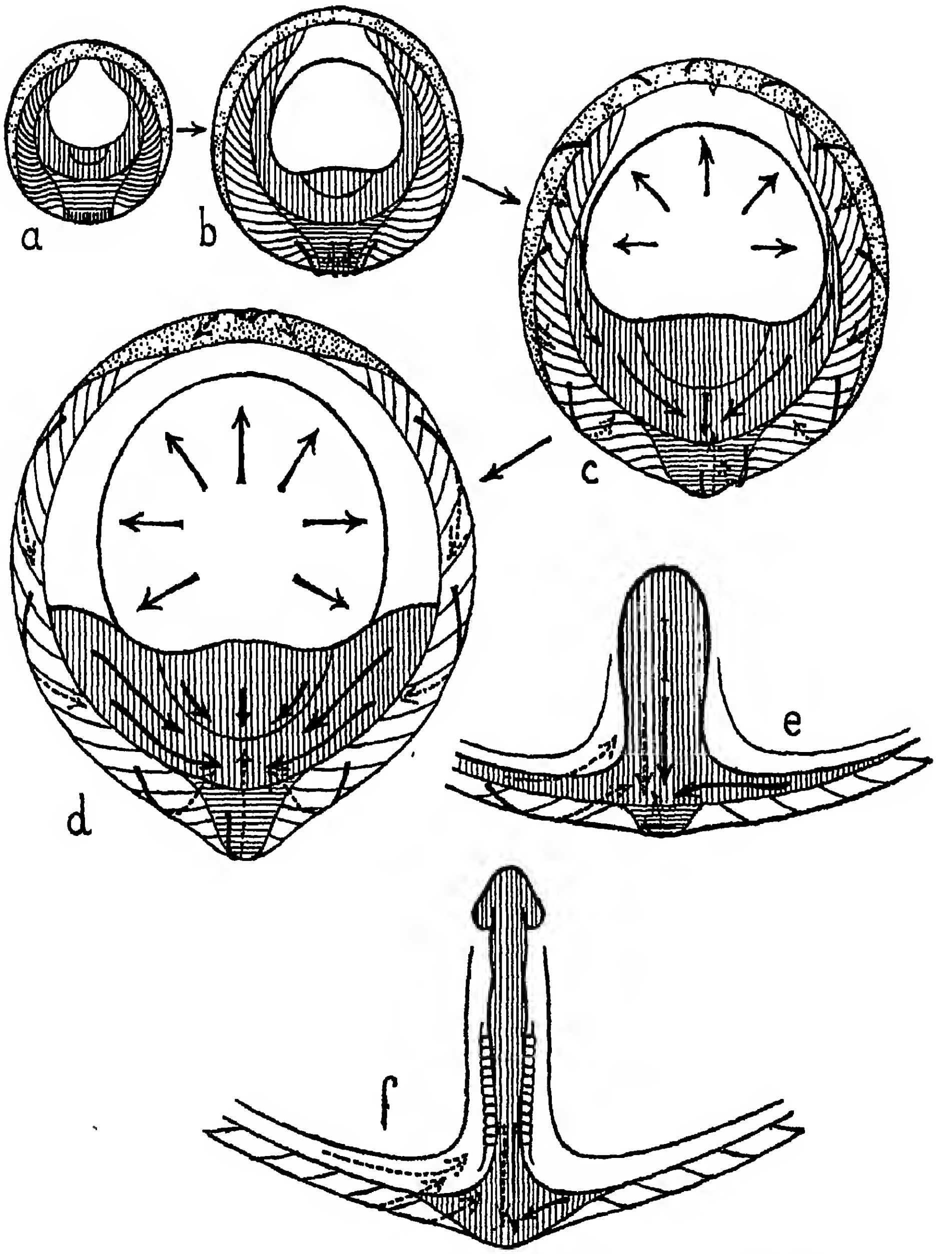


Fig. 6. Déplacements des territoires et mouvements morphogénétiques du blastodisque de Truite, vu de haut (le vitellus n'y est pas représenté). Stries verticales serrées: préchordal; stries verticales espacées: neurectoblaste, un trait y indique la limite du cerveau; traits horizontaux: chorde; traits obliques: somites; ponctuation: lames latérales; en blanc: ectoblaste; flèches pleines: déplacements en surface; flèches en tirets: déplacements en profondeur.

haut, supposée détachée du vitellus insegmenté. La continuité avec celui-ci se fait par tout le pourtour du blastodisque, contour qui coïncide avec le blastopore. Notons aussi que pour des raisons techniques, la partie caudale n'a pas pu être étudiée et qu'il n'est pas exclu que la zone somitique doive, comme chez les Amphibiens, se prolonger quelque peu au-dessus de l'ébauche chordale; des recherches devraient être reprises à ce propos chez une autre espèce. Enfin, l'entoblaste qui occupe une zone mince recouvrant la partie basse de la zone marginale n'a pas été représenté sur ces figures.

Ces réserves faites, on verra que les ébauches présentent la même configuration générale, les mêmes rapports, que les mouvements morphogénétiques sont identiques: invagination, convergence, extension, épibolie. *Il n'y a donc pas de concrescence chez les Téléostéens.* Quant à la nodosité terminale qui constitue l'extrémité postérieure de l'"écusson embryonnaire" et qui avait été considérée comme un centre de croissance par Kopsch (1904), elle n'est qu'un carrefour cellulaire: lieu de rencontre des éléments qui s'invaginent, convergent et s'étendent. La densité mitotique n'y est pas plus grande que dans les organes primordiaux déjà constitués dans les parties antérieures de l'embryon.

Et cependant que de différences dans les formes. N'insistons pas tant sur les rapports entre écusson embryonnaire et masse vitelline insegmentée, secondaires, et variables d'un Téléostéen à l'autre, selon la taille de leur masse vitelline. Mais que de différences dans les aspects microscopiques. La partie axiale d'une gastrula ou d'une jeune neurula d'Amphibien semble en effet ordonnée autour de la cavité archentérique. Mais il serait vain de rechercher une telle cavité chez un Téléostéen. Sous les somites et la chorde, l'entoblaste est représenté par une mince couche cellulaire continue. Cette lame repose directement sur le vitellus. Pour autant qu'il existerait un archentéron, il ne pourrait être représenté que par une fente toute virtuelle, sise au-dessus ou en dessous de cette lame. Mais ni l'une ni l'autre interprétation ne serait vraiment satisfaisante, car l'embryon est solidaire du syncytium vitellin sur lequel il repose; d'autre part, la fente séparant le chordomésoblaste de l'entoblaste ne communique d'aucune façon avec le blastopore. Nous voyons donc que l'œuf des Téléostéens gastrule d'une façon très analogue de celle des Amphibiens, mais sans qu'il y ait eu formation d'un archentéron. Soulignons ici un premier exemple montrant une très grande concordance dans la cinétique gastruléenne et une très grande discordance dans la morphologie de la gastrula dans deux ordres de Vertébrés. Discordance d'autant plus grosse qu'elle concerne ce point nodal de la *forme* gastrula: l'archentéron.

### 3. SÉLACIENS

Le mémoire de Vandebroek (1936, note préliminaire en 1935) est basé sur l'étude de marques colorées appliquées à *Scyllium canicula*. La répartition des ébauches sur le blastodisque de la Roussette est très semblable à celle de la Truite. Une différence toutefois: tandis que chez les Téléostéens, comme chez les Amphibiens, il existe une zone marginale annulaire et continue, chez *Scyllium* cette zone

ne constitue qu'un croissant. Il n'existe en effet ni mésoblaste présomptif du côté ventral du blastodisque, ni lèvre ventrale du blastopore chez cette espèce. Ventralement, l'ectoblaste vient au contact direct de la marge vitelline. Ce caractère n'est toutefois pas général chez les Sélaciens. On sait en effet que dans d'autres espèces, telles que *Torpedo*, il existe une lèvre ventrale du blastopore.

Les mouvements morphogénétiques analysés par Vandebroek sont les mêmes que chez les Amphibiens et les Téléostéens. Notons toutefois qu'entre la fin de l'invagination et le début de la forte extension antéro-postérieure, cet auteur note l'existence d'un temps d'arrêt. Enfin, on sait que chez les Sélaciens, contrairement aux Téléostéens, il existe un archentéron véritable.

Dans ce mémoire, Vandebroek se rallie à notre conception cinétique de la gastrulation. Toutefois, cet auteur propose de distinguer entre gastrulation et individuation des organes axiaux: la gastrulation ne serait que "la mise en place de l'entoblaste sous l'ectoblaste" (*loc. cit.* p. 530). Ceci nous paraît avoir le défaut d'être schématique, et surtout d'attacher trop d'importance à certains aspects didermiques de la gastrulation; ces aspects ne sont que purement contingents et n'ont, contrairement à ce que l'on a cru, aucune importance intrinsèque (voir plus loin).

## 4. Cyclostomes

Dès 1926, Weissenberg posait des marques colorées sur l'œuf de *Lampetra*. Ces marques au brun Bismarck ont pu ultérieurement (1929) être conservées sur coupes. On trouvera dans les quatre mémoires de 1933, 1934, 1936 de cet auteur les résultats de cette investigation. D'après ces travaux, le plan des ébauches montrerait un chordomésoblaste ramassé tout entier dans le secteur dorsal de l'œuf; ventralement, l'ectoblaste viendrait au contact de l'entoblaste. D'autre part la cinétique gastruléenne serait des plus simples; une convergence de tous les matériaux se centrant sur un petit orifice blastoporal pour constituer un canal archentérique étroit et continu à la suite d'une invagination "en doigt de gant". Ultérieurement cette paroi archentérique se scinde en composantes: chorde, mésoblaste, entoblaste. Weissenberg (1933) en conclut que le chordomésoblaste est donc chez *Lampetra* un "echter Urdarmabkömmling" (*loc. cit.* p. 408). Chez ce Vertébré primitif, le mésoblaste serait donc d'origine "gastrale", et le type entérocœlique de la formation du chordomésoblaste pourrait donc toujours être considéré comme primitif, tandis que le mode de gastrulation des Amphibiens—surtout des Urodèles—serait secondaire.

Cependant, il apparaît bien que l'étude de Weissenberg ne repose que sur des documents partiels, portant sur le début d'une gastrulation qui se poursuit bien au delà de l'apparition de la plaque médullaire (cf. Pasteels, 1939*c*). Il existe, fait que Weissenberg a complètement négligé, une lèvre ventrale chez *Lampetra* comme l'avaient déjà montré sur coupes Hatta (1891 et 1907) et de Sélys-Longchamps (1910). J'ai pu me convaincre récemment du fait que la gastrulation de *Lampetra* ne diffère de celle des Amphibiens que par la chronologie. Il y existe, comme chez

les Amphibiens, un anneau marginal chordomésoblastique continu. Je ne puis, faute de documents suffisants, présenter un plan des ébauches de la jeune gastrula pouvant remplacer la version, certainement inexacte, de Weissenberg. Mais la fig. 7 nous montre le plan de la jeune neurula. L'œuf y est vu de profil (*a*), de haut (*b*) et de l'arrière (*c*). On y verra *tout autour* du blastopore un anneau chordomésoblastique *continu*, se composant de chorde (pointillés) en avant, de somites sur les côtés, de mésoblaste ventral (tirets) en arrière. Les mouvements de convergence, d'extension et d'invagination y sont représentés par des flèches pleines en surface, pointillées en profondeur. L'invagination du mésoblaste ventral qui se réfléchit

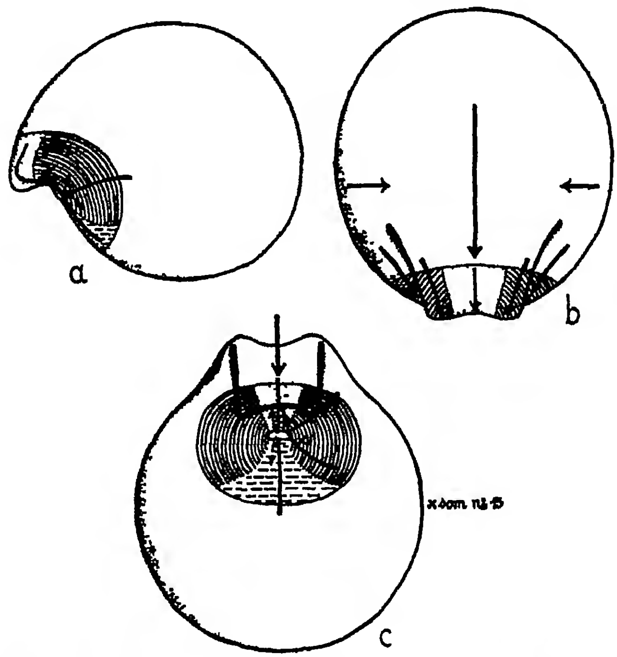


Fig. 7. Plan des ébauches et mouvements morphogénétiques au stade plaque médullaire chez *Lampetra Planeri*. *a*. Profil; *b*. vue de haut; *c*. vue postérieure. Flèches pleines: mouvements en surface; flèches pointillées: mouvements en profondeur (chordomésoblaste invaginé). Pointillé: chorde; traits parallèles: somites; tirets: mésoblaste ventral.

entre l'ectoblaste et l'entoblaste *ne débute qu'à ce stade*, et c'est sans doute la raison pour laquelle cette lèvre ventrale a échappé à Weissenberg. Remarquons la quantité considérable de matériel qui doit encore s'invaginer, malgré l'apparition de la plaque médullaire: aux abords immédiats du blastopore nous trouvons encore en surface le 15ème somite, or le tronc de l'Amnocète en possède plus de 75. Cette gastrulation se poursuit d'ailleurs bien plus tard et ne se termine que lorsque le tube neural, fermé depuis longtemps, s'est considérablement allongé et que les vésicules oculaires sont déjà apparues. A ce point de vue, la gastrulation de *Lampetra* ressemble fortement à celle des Téléostéens, chez lesquels nous voyons aussi (fig. 6) la partie antérieure du corps prendre forme alors que la majeure partie du corps embryonnaire est encore en voie d'invagination. Il est d'ailleurs remarquable de

voir que chez *Bdellostoma*, ce Cyclostome à œufs méroblastiques, les aspects de la gastrulation publiés par Bashford Dean (1899) ressemblent de façon étonnante à ceux de la Truite. La raison nous en paraît simple: tant les Téléostéens que les Cyclostomes ont un corps pisciforme, c'est-à-dire très allongé, à nombre considérable de segments.

Les deux points principaux qui méritent d'être relevés, du point du vue général, sont les suivants. (*a*) La cinétique gastruléenne est la même que chez les Amphibiens et n'en diffère que par la chronologie; cette simple discordance chronologique entraîne toutefois de grandes différences dans les formes gastruléennes: chez les Amphibiens, la progression de l'invagination dans le sens dorso-ventral est rapide et se fait avant que la masse vitelline ne soit englobée; le blastopore sera dilaté par un "bouchon vitellin"; chez les Pétromyzontes, cette progression est lente; au moment où la lèvre ventrale se constitue, le vitellus a déjà basculé dans le plancher archentérique; le blastopore reste toujours petit. Chez les Amphibiens, dès le début de la gastrulation, du moins après l'invagination des matériaux céphaliques, il existe un "manteau mésoblastique" (cf. fig. 3) en grande partie distinct de la cavité archentérique. Chez les Pétromyzontes, ce manteau n'est que d'apparition plus tardive. (*b*) Il n'en est pas moins vrai que ce manteau existe chez les Pétromyzontes, qu'une partie du mésoblaste n'est ou n'a jamais été en rapport avec la cavité archentérique, et comme le chordomésoblaste constitue manifestement un tout indissociable, *la conception entérocœlique n'est donc pas applicable aux Cyclostomes. La formation blastoporale, "péristomale", du mésoblaste, telle que l'a décrite Vogt* (1929*a*) *chez les Amphibiens est une règle générale, applicable à tous les Vertébrés, même les plus primitifs.*

## 5. Prochordés

La gastrulation de l'Amphioxus est d'un intérêt primordial. En effet, elle a toujours été considérée comme réalisant par excellence le type de la gastraea; d'autre part, entre toutes les gastrulas de Chordés, elle est la seule où l'on puisse observer des aspects évidents d'entérocœlie. Il semble bien toutefois que les premières études en aient été assez superficielles, car le mémoire de Conklin est apparu en 1932 comme une véritable révélation. Les observations minutieuses de cet auteur, et sa longue pratique du "cell-lineage" ont pu démontrer que le mode de gastrulation de l'Amphioxus est, à part le degré de cellularisation du germe, presque identique à celui de ces autres Prochordés que sont les Ascidies.

La gastrulation des Ascidies qui avait déjà fait l'objet des travaux classiques de Conklin (1905) a été reprise récemment par Vandebroek, utilisant chez *Ascidiella*[1] deux méthodes: les marques colorées et le déplacement des blastomères greffés en diverses combinaisons. Le plan de Vandebroek concernant le stade VIII —et qui se subira plus de modifications jusqu'à la gastrulation—est reproduit sur la fig. 8*c*. Nous y voyons trois croissants caractéristiques: neural et chordal du côte

[1] Ces travaux sont actuellement en voie de publication; les résultats m'en ont été communiqués de vive voix par mon collègue et ami, le Dr G. Vandebroek, que je tiens à remercier bien vivement.

dorsal, et mésoblastique du côté ventral. Notons ce point important: alors que chez les Vertébrés, la zone marginale chordo-mésoblastique est continue, chez *Ascidiella*, *la zone marginale est discontinue*, se composant d'un croissant chordal dorsal, d'un croissant mésoblastique ventral, *sans aucun contact entre les deux*. La cinétique gastruléenne, grâce aux deux méthodes employées par Vandebroek, a pu être analysée dans ses moindres détails. Il est à souligner que ces observations complètent et précisent, mais n'infirment en aucune manière les résultats du "cell-lineage" de Conklin. L'invagination est brutale et se réalisera par un véritable aplatissement de la blastula en une "placula". Le pli de séparation entre le feuillet externe et le feuillet interne apparaît donc de façon très précoce. On observe ensuite une convergence de matériaux ectoblastiques du côté dorsal produisant une inflexion en crochet, au niveau de la partie dorsale du germe. La progression de l'épibolie du feuillet externe, se combinant à la convergence dorsale, va fermer progressivement le blastopore, primitivement béant.

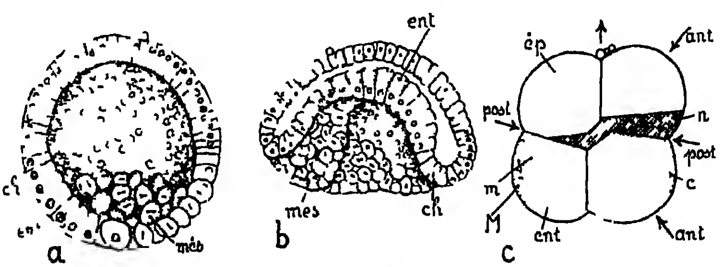


Fig. 8. Coupe optique (*a*) d'une blastula, (*b*) d'une gastrula d'Amphioxus, d'après Conklin (1932). *ch.* chorde; *ent.* entoblaste; *més.* mésoblaste. (*c*) Plan des ébauches de l'œuf d'*Ascidiella*, au stade VIII blastomères d'après G. Vandebroek (in Dalcq, 1938*b*). *ant.* antérieur; *post.* postérieur; *n.* neurectoblaste; *c.* chorde; *ent.* entoblaste; *M.* myoblastes; *m.* mésenchyme; *ép.* épiblaste.

Dorsalement, la convergence s'accentue et se combine à un mouvement d'extension, apparaissant d'abord dans le matériel ectoblastique, puis dans le matériel chordal. Cette extension contribue à constituer le dos et à reporter le blastopore vers l'arrière. Pendant ce temps, le matériel profond, comme on le verra sur la fig. 8*b* concernant l'Amphioxus (Conklin), se met en place. Tandis que la plaque entoblastique, composée de grosses cellules, s'applique sur le fond du blastocœle, le croissant mésoblastique (petites cellules rondes, *més.*) qui se trouvait au rebord ventro-latéral du blastopore, se replie de telle façon que ses deux bras viennent se placer parallèlement et constituer les deux "gouttières mésoblastiques" au niveau de la voûte archentérique; c'est entre ces gouttières mésoblastiques que viennent s'intercaler les cellules chordales qui, à la suite du mouvement d'extension-convergence, viennent se disposer en file linéaire.

Deux conclusions nous paraissent devoir être relevées.

(*a*) A certains égards, la cinétique gastruléenne est très semblable à celle des Vertébrés. Nous y retrouvons l'invagination, l'épibolie, l'extension et la convergence. La mise en place de la chorde et la formation du système nerveux sont directement homologables aux mêmes processus des Vertébrés. Notons cependant une grosse différence dans le comportement du mésoblaste. Nous avons vu en effet

que, chez les Vertébrés, le mésoblaste somitique se trouve placé, dès le début du développement, sur les flancs de l'ébauche chordale, et que sa cinétique est, quoiqu'à un moindre degré, analogue à celle de la chorde. On peut affirmer, et toutes les investigations expérimentales le prouvent d'ailleurs, que les somites des Vertébrés constituent une sorte de "parachorde", faisant partie du même système que la chorde et n'en différant vraisemblablement que par des variations quantitatives. Or, chez les Ascidies, nous voyons qu'il existe dès la blastula un croissant mésoblastique *ventral*, autonome. De par cette situation ventro-latérale d'emblée, le mésoblaste vient se ranger directement sur les flancs de la chorde, sans devoir converger. D'ailleurs, les expériences d'isolement et de translocations de blastomères de Vandebroek montrent que ce matériel mésoblastique possède un très grand pouvoir d'extension, mais *aucun* pouvoir de convergence. On a nettement l'impression, que l'analyse de l'œuf insegmenté telle que l'a réalisée Dalcq (1938*a*) par ses expériences de mérogonie ne peut que renforcer, que le croissant mésoblastique des Prochordés n'a pas d'équivalent réel chez les Vertébrés, où il aurait pu être remplacé par un autre mésoblaste dépendant du système chordal.

(*b*) A mon avis, tant les travaux de Conklin que ceux de Vandebroek permettent de conclure que, même chez les Prochordés, l'entérocœlie n'a aucune valeur réelle. Il n'est en effet plus possible d'affirmer qu'il existe à un moment donné une gouttière chordale et des poches cœlomiques, qui se détacheraient secondairement d'un archentéron continu: dès la blastula, on peut, comme on le voit bien sur la fig. 8*a* concernant l'Amphioxus (d'après Conklin), distinguer déjà par la taille, la disposition, la colorabilité des cellules, les zones chordale, mésoblastique, entérique. Les isolements de blastomères réalisés par Vandebroek précisent que ces territoires possèdent dès le stade VIII des potentialités différentes et autonomes. Le "feuillet" profond du stade didermique des Prochordés est en réalité une mosaïque, un assemblage provisoire d'élements différenciés déjà au moment où ils viennent constituer provisoirement une partie du revêtement archentérique. S'il existe un stade didermique chez les Prochordés, ce n'est non pas en souvenir d'une gastraea ancestrale bien hypothétique, mais parce que la disposition bien simple de la blastula unicellulaire, facilite l'invagination d'emblée de tous les éléments profonds, si différents soient-ils.

## 6. Reptiles

Les travaux classiques laissaient supposer qu'il doit exister chez les Reptiles un type de gastrulation intermédiaire entre celui des Vertébrés inférieurs et des Amniotes homéothermes, car on y décrivait à la fois un blastopore aboutissant à un canal "archentérique" et une "plaque" ou une "ligne primitive". Il était toutefois difficile de comprendre comment ces deux formations se combinaient et quels étaient leurs rôles respectifs dans l'ensemble de la gastrulation; d'autre part, pour montrer à quel point l'étude des seules coupes sériées rendait la gastrula des Reptiles difficile à interpréter, il suffira de rappeler que les auteurs ne s'accordaient même pas sur l'emplacement qu'il faut assigner à la lèvre ventrale du blastopore, ni sur la valeur du canal soi-disant "archentérique".

La combinaison de l'étude microscopique et des marques colorées sur l'œuf vivant a été possible chez une Tortue, *Clemmys leprosa* Schw., en récoltant les œufs dans les conduits maternels (Pasteels, 1937*a*).

Un des problèmes les plus discutés est celui de l'origine de l'entoblaste. On sait en effet que, chez les Reptiles comme chez tous les Amniotes, il se forme d'abord dans la partie embryonnaire du germe deux feuillets primaires et ce n'est qu'ensuite que le chordomésoblaste issu du feuillet superficiel se met en place. Au stade de la formation de l'embryon didermique, il n'est pas encore possible d'appliquer des marques colorées chez *Clemmys*, mais heureusement les images microscopiques y sont exceptionnellement claires.[1]

La partie segmentée (blastodisque) de l'œuf de *Clemmys* s'étale quelque temps avant la gastrulation, en une couche pratiquement unicellulaire appliquée intimement sur la surface du vitellus. Ce n'est que de-ci de-là que l'on trouve quelques cellules éparses en profondeur. *Au centre* de ce blastoderme étalé se différencie l'aire embryonnaire (ovales périphériques de la fig. 9). La majeure partie des cellules, jusqu'ici aplaties, y deviennent cylindriques (ovales internes de la fig. 9); en même temps, une liquéfaction du vitellus sous-jacent crée la "cavité sous-germinale". Vers l'extrémité postérieure de l'aire embryonnaire, mais *à l'intérieur de celle-ci*, c'est-à-dire à la limite postérieure de l'"écusson" à cellules hautes (cf. fig. 9*A*), apparaît un sillon blastoporal arciforme. Des coupes longitudinales y montrent une invagination très active de cellules qui se portent vers l'avant, pour y constituer le feuillet profond. Dans celui-ci sont aussi englobées les quelques cellules qui se trouvaient déjà en profondeur. Il est totalement exclu que celles-ci puissent constituer la source exclusive, voire principale, de l'entoblaste; elles sont beaucoup trop rares et ne montrent par ailleurs pas de mitoses. Au niveau du blastopore on assiste également à une invagination vers l'arrière: les cellules s'y accumulent dans l'amas dit de la plaque "primitive", mais qu'il vaut mieux qualifier de plaque blastoporale. Cet amas constitue une sorte de réserve d'où sont issues les cellules entoblastiques de la partie postérieure de l'embryon. C'est d'ailleurs au sein de cet amas que Risley (1933) a noté l'origine des gonocytes primordiaux chez la Tortue *Sternotherus*. Notons en passant un rapprochement qui mérite d'être signalé: chez les Amphibiens Anoures, Bounoure (1934) a décelé la présence d'un "déterminant génital" au centre de la masse entoblastique vitelline; ce plasme spécialement colorable va, au cours de la segmentation, se loger au plancher du blastocœle; les cellules qui le contiennent deviennent gonocytes primordiaux.

On ne s'accorde toutefois pas à décrire la formation de l'entoblaste de la même façon chez les autres Reptiles. La plupart des auteurs y ont vu une délamination, c'est-à-dire une séparation sur place des éléments superficiels et profonds issus de la segmentation par creusement d'interstices intercellulaires se réunissant progressivement. C'est une telle thèse que défend actuellement Peter (1934*b*, 1935, 1938*c*), en se basant sur des préparations de Lézard et de Caméléon. Comme le montre surtout son dernier mémoire, il existe incontestablement chez ces

[1] Faute de place, je n'ai pu y consacrer l'illustration nécessaire pour emporter la conviction semble-t-il (cf. Peter, 1938 *c*). Cette lacune sera comblée dans un mémoire ultérieur.

espèces une délamination séparant à un moment donné un feuillet superficiel d'un feuillet profond. Mais s'agit-il de la formation de tout l'entoblaste ? Nous nous permettons d'en douter. Ce regroupement cellulaire ne serait-il pas plutôt l'analogue d'une sorte de blastulation et la gastrulation proprement dite ne commencerait-elle pas par l'invagination de cellules *entoblastiques* au niveau du

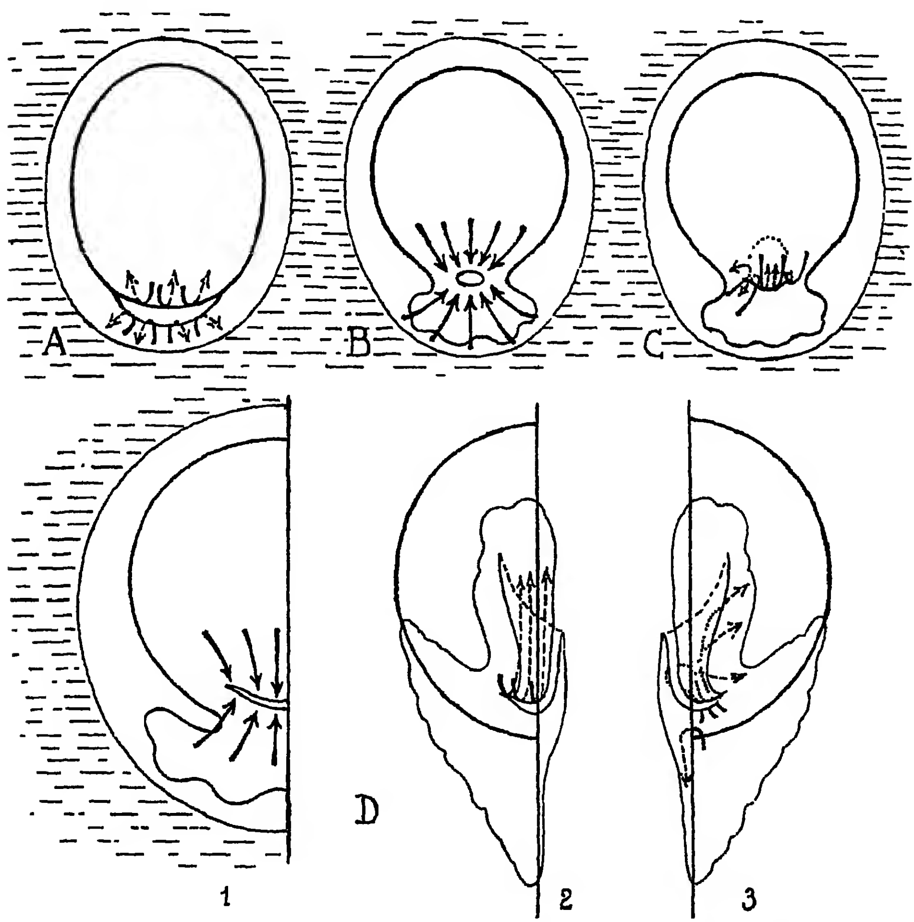


Fig. 9. Schéma des mouvements cellulaires de la gastrulation de *Clemmys leprosa*. Tirets : aire extra-embryonnaire. $D_2$ et $D_3$ sont supposés vues en perspective à 45° par la tranche de section sagittale; les autres figures sont vues de haut. Flèches pleines : mouvements en surface; flèches en tirets : mouvements dans un premier plan profond; flèches ponctuées : mouvements dans un second plan profond.

blastopore. Celles-ci viendraient se mêler au feuillet vitellin sous-jacent et constituer peut-être la partie axiale, embryonnaire proprement dite de l'entoblaste. Cette supposition est formellement contredite, il est vrai, par Peter qui croit que la masse cellulaire issue du blastopore reste absolument distincte de l'"entoblaste" issu de la délamination. Mais sur ce point, la démonstration de l'auteur allemand

ne me paraît pas convaincante et il me paraît bien difficile de devoir admettre que la formation de l'entoblaste serait de nature différente chez les divers Reptiles. La question mérite de toute façon nouvel examen.

Pour revenir à la Tortue, quand le stade didermique s'est constitué, on voit le blastopore devenir plus etroit. Les matériaux situés tout autour de lui y convergent (fig. 9 *B*), puis s'y invaginent (fig. 9 *C*) pour constituer un canal *chordo-mésoblastique* (c archentérique ou blastoporal des auteurs). Dès ce stade, les mouvements cellulaires ont pu être suivis par des marques colorées. Convergence et invagination continuent au fur et à mesure que le canal s'allonge. La paroi supérieure du canal sera constituée par des matériaux situés primitivement en avant du blastopore; après s'être réfléchis dans la voûte du canal (fig. 9 $C$, $D_2$), ils vont constituer la chorde; ses parois latérales qui vont former les somites (voir plus loin) proviennent de matériaux situés primitivement sur les côtés du blastopore; son plancher dérive des matériaux postérieurs et postéro-latéraux qui franchissent le talus blastoporal postérieur.

A première vue, on serait tenté d'attribuer à ce plancher une valeur entoblastique en considérant le canal comme un véritable archentéron. Mais une telle interprétation doit être délibérément rejetée: le canal est tapissé de toutes parts de chordo-mésoblaste; son plancher n'est pas entoblastique mais mésoblastique. En effet, sur coupes sagittales, on verra que les cellules postérieures qui franchissent le versant postérieur (lèvre ventrale) du blastopore, après avoir circulé quelque temps dans le plancher de la partie postérieure du canal, se réfléchissent vers l'arrière pour constituer du mésoblaste extra-embryonnaire (fig. 9 $C$, $D_3$). De même, des coupes transversales, comme nous les voyons sur la fig. 10, nous montreront que les cellules du plancher du canal émigrent vers les côtés pour constituer la partie la plus latérale du *mésoblaste* (extra-embryonnaire et lames latérales). Ces coupes proviennent du même embryon et s'étagent d'arrière en avant; la morphogénèse progressant d'avant en arrière, elles représentent donc des aspects de plus en plus avancés. Sur la fig. 10 *a* nous voyons le canal complet dont les parois sont comprises entre le haut épithélium ectoblastique et le mince feuillet, endothéliforme, mais continu, entoblastique; en 10 *b* nous apercevons une migration intense des cellules du plancher vers les côtés, aboutissant à un amincissement très accentué de ce plancher; en 11 *d* nous voyons que ses deux lèvres latérales elles-mêmes se sont appliquées sous les parties latérales de l'ectoblaste.

L'entoblaste constituait un feuillet mince mais continu au-dessus duquel le canal est venu s'insinuer. La migration latérale des cellules mésoblastiques du plancher semble cliver ce feuillet mince, et de fait, plus tard, au moment de la séparation des organes primordiaux, on retrouve un feuillet entoblastique unicellulaire, fendu sur la ligne médiane et se terminant par deux bords libres au niveau de la chorde. La disposition typique de la voûte archentérique est donc réalisée par un processus secondaire. Il est impossible d'affirmer que l'entoblaste définitif provienne uniquement du feuillet entoblastique primaire et que certains éléments du plancher du canal ne s'y soient pas ajoutés. Cette dernière éventualité reste possible, mais il est bien certain que cette participation du plancher du canal à l'entoblaste ne

pourrait être que très réduite; on voit en effet passer la grosse masse de ces éléments du plancher dans le mésoblaste latéral.

Les mouvements morphogénétiques, à ces stades déjà avancés de la gastrulation, seront schématisés sur la fig 9*D*. On y représente des gastrulas supposées coupées sagittalement. La première est posée à plat, et montre les formes extérieures, et indique en flèches pleines les mouvements du feuillet superficiel. Les deux autres sont supposées vues en perspective, la face supérieure et la tranche de section étant vues à 45°. Sur cette section, on apercevra la lumière du canal chordo-mésoblastique; en pointillé est indiquée la limite antérieure de son plancher en voie d'épanouisse-

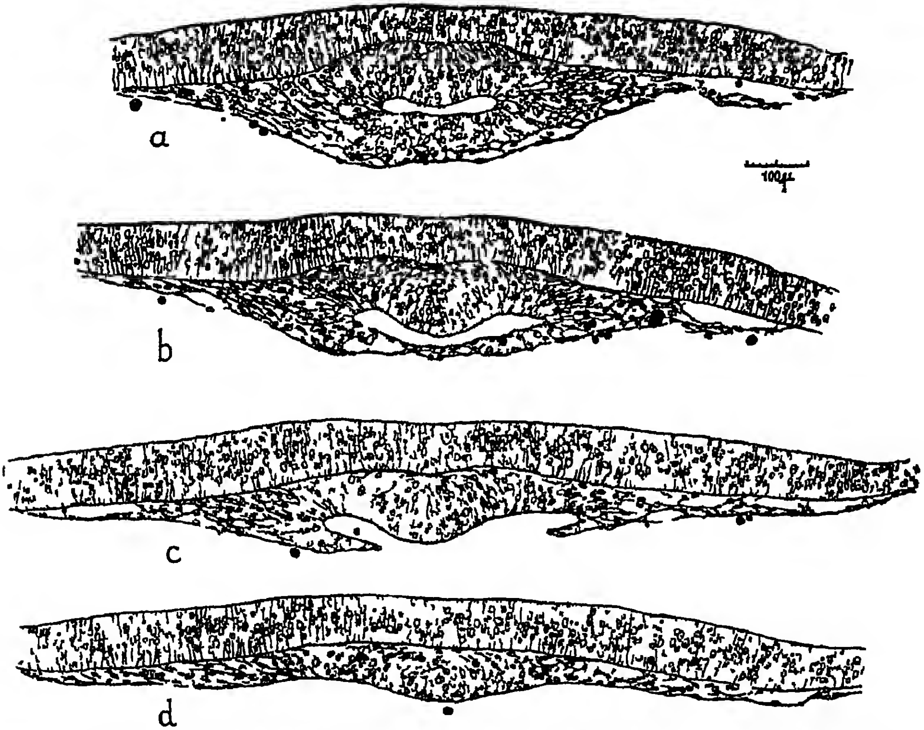


Fig. 10. Quatre coupes transversales passant par quatre niveaux, s'étageant d'arrière en avant d'un même embryon de *Clemmys leprosa*.

ment; un trait curviligne, concave en dehors et rejoignant le blastopore, indique la limite latérale de la lumière du canal; le trait sinueux suit (d'après une reconstruction graphique) la marge périphérique du mésoblaste latéral et extra-embryonnaire. Les flèches en tirets indiquent les mouvements cellulaires dans le second plan cellulaire—donc soit dans le plafond du canal ou dans le mésoblaste latéral; les flèches pointillées figurent les mouvements dans le troisième plan cellulaire: le plancher du canal. Nous assistons donc à des mouvements d'*invagination*, de *convergence dorsale*, de *divergence ventrale*; aussi à des mouvements d'*extension*; ceux-ci sont toutefois surtout accentués au moment de l'élongation de l'embryon qui coïncide avec l'apparition de la plaque médullaire.

Cette revue de la gastrulation d'un Reptile nous permet les conclusions suivantes:

(1) Les mouvements morphogénétiques sont les mêmes que chez les Amphibiens, leur chronologie est un peu différente toutefois: l'invagination de l'ensemble de l'entoblaste y est plus précoce; l'invagination du chordomésoblaste n'est pas progressive, mais synchrone; l'extension est plus tardive.

(2) Contrairement à ce qu'avait cru O. Hertwig (1906) d'après les données de son élève Gerhardt (1901) sur la Couleuvre, il n'y a pas d'entérocœlie chez les Reptiles. Les "poches cœlomiques" ne sont que les parties latérales du canal chordomésoblastique, déprimé en son milieu par la saillie chordale; replis temporaires disparaissant au moment de la perforation et de l'étalement du plancher, ils n'ont aucun rapport avec le cœlome véritable.

(3) Une fois de plus, malgré la très grande similitude de la cinétique, les formes gastruléennes se montrent irréductibles les unes aux autres: le canal constitué par l'invagination des cellules au niveau du blastopore n'est pas—malgré toutes les apparences—un archentéron véritable, puisqu'il est tapissé de toutes parts de chordomésoblaste et que sa lumière n'est donc en rien archentérique.

(4) C'est sur la foi aussi de formes et d'aspects, que l'on avait cru devoir décrire une "plaque primitive", même une "ligne primitive" chez les Reptiles. Or, cette forme particulière de blastopore à laquelle on doit réserver ce nom est spéciale aux Oiseaux et aux Mammifères et n'existe pas chez les Reptiles.

(5) Tandis que chez les Anamniotes, on pouvait suivre des marques colorées depuis la blastula jusqu'à la morphogénèse primordiale et établir un véritable *plan* des ébauches, chez les Reptiles, cette opération n'est plus possible. Les marques sont trop fugaces et ne peuvent indiquer que la direction des mouvements cellulaires. Mais connaissant le point de départ des matériaux, leurs directions, et leur lieu d'arrivée, on peut assez facilement dresser un *schéma* des ébauches montrant leur disposition générale, sans cependant que leurs contours puissent être tracés avec exactitude. Ce schéma, pour l'embryon didermique des Reptiles, est très analogue à celui qui concerne les Oiseaux au même stade et qui est représenté sur la fig. 11 A. Les rapports généraux des croissants neural, chordal, la disposition des somites y sont les mêmes que chez les Anamniotes. Mais une grosse différence apparaît en ce qui concerne la disposition du blastopore. Chez un Anamniote méroblastique, tel qu'un Téléostéen ou un Sélacien, le blastopore suit les contours du blastodisque et se trouve donc à la marge qui le sépare du vitellus insegmenté. Tandis que sur la fig. 11 A nous voyons que la zone blastoporale qui se trouve au centre du mésoblaste (immédiatement en arrière de la zone quadrillée représentant le mésoblaste céphalique) est *à l'intérieur de l'aire embryonnaire*. Cette aire est entourée d'ectoblaste extra-embryonnaire (en hachures sur la figure): c'est celui-ci qui se trouve à la marge du blastodisque et du vitellus.

(6) Au point de vue cinétique, cette différence est importante. Car, en effet, chez un Téléostéen, la masse vitelline est englobée progressivement par le blastopore: gastrulation et enveloppement du vitellus n'y font qu'un. Mais chez un Amniote, le blastopore n'intervient en rien dans le processus; c'est le bord d'enve-

loppement ectoblastique qui enfouit le vitellus. Cette épibolie ectoblastique se fait indépendamment des autres mouvements gastruléens. Aussi l'enveloppement du vitellus peut être réalisé dès le tout début de la gastrulation, comme chez le Caméléon (Peter, 1934*b*), ou bien encore comme c'est souvent le cas, bien après. Les rapports de cette épibolie extra-embryonnaire et de la formation de l'amnios ont été envisagés par Dalcq (1937, 1938*b*).

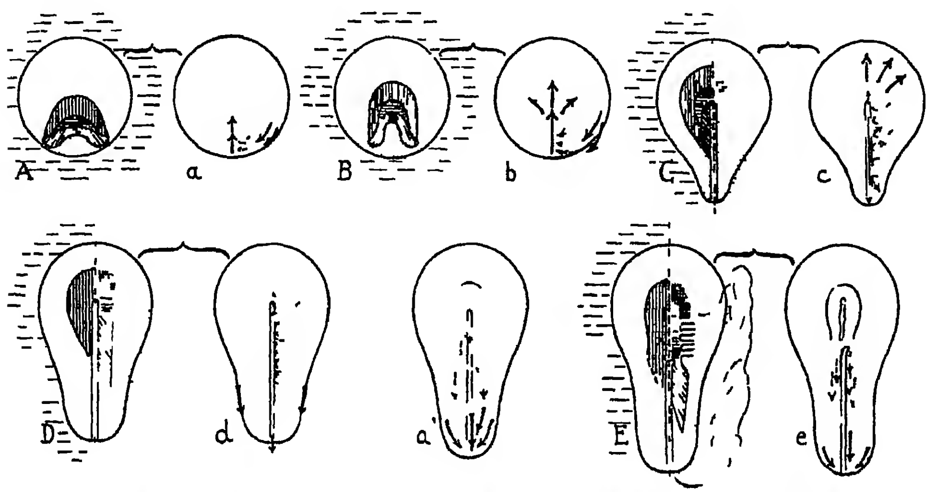


Fig. 11. Plan des ébauches (A, B, C, D, E) et mouvements cellulaires (*a*, *b*, *c*, *d*, *e*) de stades correspondants du développement du Poulet. L'aire extra-embryonnaire est vue de haut. Tirets: aire extra-embryonnaire. Dans les figs. C, D, et E, le feuillet superficiel est supposé enlevé dans la moitié droite. Flèches pleines: mouvements simultanés de toutes les parties du germe; flèches en tirets: mouvements morphogénétiques propres à l'ectophylle. Hachures verticales: neurectoblaste; hachures horizontales: chorde; grillage: préchordal; stries obliques: somites; pointillé: mésoblaste ventral et extra-embryonnaire.

## 7. Oiseaux et Mammifères

La gastrulation des Oiseaux et des Mammifères peut être envisagée simultanément. A vrai dire ce n'est que chez les Oiseaux que la technique des marques colorées a pu apporter des documents nouveaux. Mais certains Mammifères gastrulent exactement comme les Oiseaux; l'exemple le plus frappant est la Porc, si bien étudié par les reproductions graphiques de Streeter (1927). D'autres, comme les Monotrèmes, ont une gastrulation purement reptilienne. La plupart, et ici le besoin des marques colorées se fait encore sentir, présentent un type intermédiaire entre Reptiles et Oiseaux, possédant à la fois un canal "blastoporal" et une ligne primitive (en ce qui concerne les travaux récents, cf. Hamilton, 1937; Jolly & Férester-Tadié, 1936).

(1) La formation de l'embryon didermique est moins claire encore que chez les Reptiles. D'après la plupart des auteurs, l'entoblaste primaire résulterait d'une simple délamination. En 1909 paraît le mémoire de Patterson dans lequel cet auteur étudie de très jeunes œufs de Pigeon, prélevés dans les conduits maternels, mais d'âge connu grâce à une particularité de la ponte chez cet Oiseau. Cette étude,

abondamment illustrée, semble impeccable et vient donner vigueur nouvelle à la thèse ancienne de M. Duval: l'entoblaste se constituerait par enroulement à la marge postérieure du blastodisque. Mais une contribution remarquable due récemment à Peter (1938*c*) a permis à cet auteur de démontrer que la conception de Patterson est entièrement erronée. Pour reprendre la forte mais juste parole de Peter (1938*c*, p. 447): "Sie ist zu verwerfen, und soll aus dem Schriftum verschwinden."

En 1935 Mehrbach, élève de Gräper, décrit à la surface du blastoderme non incubé et coloré préalablement au rouge neutre des plis irréguliers (aspects de "chagrin"). Au fond de ces plis, disséminés sur tout le blastoderme, mais plus nombreux dans sa partie postérieure, des cellules isolées quitteraient le feuillet superficiel pour venir constituer peu à peu le feuillet profond. Pour ma part, constatant sur des œufs fixés au début de l'incubation de ces images de "polyinvagination" (cf. Pasteels, 1937*b*: fig. 4, p. 399), je me croyais en droit de confirmer l'interprétation de Mehrbach. J'avais tort. Ces images d'enfouissement de quelques cellules de la superficie vers le feuillet profond ne concernent que les stades tardifs de la formation de l'entoblaste, ceux que l'on observe sur des œufs non incubés mais récoltés sans précautions spéciales. Dans ces conditions, la grosse partie de l'entoblaste primaire est déjà formée. Mon erreur était de supposer que la "polyinvagination" observée à ce moment n'était que la continuation d'un processus débutant beaucoup plus tôt.

Les deux mémoires récents de Peter (1938 *b*, *c*) ont mis les choses au point. Fixant des œufs de Poule au sortir du nid, ou récoltant des œufs de Pigeon dans les voies maternelles, utilisant tant l'étude des coupes que les marques colorées, cet auteur montre de façon indubitable que l'embryon didermique des Oiseaux se forme par délamination: de petits espaces intercellulaires qui bientôt confluent, séparent peu à peu la couche profonde de la couche superficielle. Au moment où paraissaient ces mémoires, j'étudiais pour ma part une série d'œufs de Cane. Ces œufs sont pondus plus tôt que chez la Poule: les images que j'y ai observées concordent en tous points avec celles décrites par Peter. Mais comme nous l'avons fait remarquer chez les Reptiles, s'agit-il bien de la formation du véritable *entoblaste* de l'embryon définitif? Ne s'agit-il pas plutôt d'un arrangement cellulaire prégastruléen, d'une blastulation? L'interstice apparu ainsi au cours de la segmentation entre les cellules profondes et les cellules superficielles aurait la valeur d'un blastocœle. Ceci serait assez satisfaisant puisque le ligne primitive s'immisce entre les deux couches de même qu'un archentéron d'Amphibien s'invagine dans le blastocœle.

Dans ce cas, le véritable entoblaste se formerait ultérieurement. Deux possibilités sont à retenir. (*a*) La polyinvagination: si Peter (1938*b*) a bien montré que les plis décrits par Gräper et Mehrbach sous le nom de "chagrin" sont bien des artefacts, il n'en est pas moins vrai que l'on assiste après la délamination à une invagination sporadique, mais qui semble difficilement contestable (cf. Pasteels, 1937*b*, fig. 4, p. 399), sous forme de cellules isolées. Et ceci concorde d'ailleurs avec certaines observations chez les Mammifères. Chez la plupart de ceux-ci, on s'ac-

corde, faute de mieux, à y décrire de la délamination; mais chez certains la poly-invagination est évidente. Chez les Marsupiaux en effet (Hill, 1910; Kerr, 1934), dans la paroi unicellulaire de la vésicule blastodermique, certaines cellules se distinguent par leurs affinités tinctoriales; puis on les voit s'enfoncer de-ci de-là pour venir constituer un feuillet profond et continu. La même évolution a été décrite chez le Tatou par Patterson (1913). (*b*) La ligne primitive: les travaux de Hunt (1937) ont montré que des cellules provenant de la ligne primitive viennent se mêler au feuillet entoblastique sous-jacent. Des documents sur le sort ultérieur de ces cellules manquent encore; il n'en est pas moins probable, contrairement à ce que j'avais cru précédemment, qu'une partie au moins de l'entoblaste embryonnaire puisse provenir de la ligne primitive, véritable blastopore comme nous le verrons.

Cette question difficile de l'entoblaste chez les Amniotes n'est donc pas encore résolue. Il ne paraît nullement que la solution doive se trouver dans les observations récentes mais assez extraordinaires de Jacobson (1938*a*). Cet auteur prétend avoir trouvé un archentéron(?) entoblastique, précédant la ligne primitive, sous la forme d'un tube partant de l'ectoblaste. L'auteur n'indique d'ailleurs pas s'il s'agit d'un aspect régulier d'un endroit bien localisé de *tous* ses embryons ou d'une trouvaille fortuite. Je pense qu'il doit s'agir de cette dernière éventualité car je n'ai jamais rien vu de pareil dans mes coupes, si ce n'est sous forme de plissements dûs à la fixation.

(2) Lorsque le stade didermique est constitué, au sein de l'aire embryonnaire arrondie apparaissent les premiers mouvements cellulaires décelables par les marques colorées. A première vue, ils semblent assez complexes en raison de la *combinaison de deux ordres de déplacements cellulaires*: les mouvements morphogénétiques proprement dits et des remaniements d'ensemble de l'aire embryonnaire affectant de la même façon les deux feuillets primaires. Gräper (1929) et Wetzel (1929), qui les ont analysés en premier lieu, n'ont pu faire cette distinction et les déplacements qu'ils ont observés dans le feuillet superficiel sont donc la résultante de ces deux composantes hétérogènes: on comprend alors pourquoi ils apparaissent assez étranges et difficilement réductibles aux mouvements des autres Vertébrés.

C'est en plaçant des repères sur la membrane vitelline—et non sur l'embryon même, puisqu'il change de forme—que l'on peut observer les déplacements simultanés des deux feuillets (Pasteels, 1937*b*). Récemment, Peter (1938*b*) en a donné confirmation d'après l'étude de marques colorées conservées sur coupes. Ces mouvements sont schématisés par des flèches *pleines* sur la fig. 11. Dans l'aire embryonnaire encore arrondie (fig. 11*a*) on observe une poussée de matériaux postéro-médians vers l'avant; en même temps les éléments latéro-postérieurs s'infléchissent vers le dedans et vers l'arrière. Les mêmes mouvements se précisent au moment de la première apparition de la ligne primitive (fig. 11 *b*, *c*); on aperçoit à présent que c'est le matériel de la ligne primitive qui s'allonge tandis que les territoires situés en avant d'elle s'épanouissent vers l'avant et les côtés. Une poussée de la ligne primitive vers l'arrière vient s'ajouter au fléchissement postérieur de la marge postéro-latérale et c'est ainsi que le germe devient piriforme (fig. 11 *c*, *d*). À ce stade, les mêmes mouvements persistent et aboutissent à une aire embryonnaire

plus allongée, une ligne primitive plus longue aussi, et dont l'extrémité antérieure se rapproche de la marge de l'aire embryonnaire (fig. 11 *d*). A partir de ce stade, les mouvements se restreignent à la partie postérieure de l'aire (fig. 11 $d^1$, *e*).

Sur ces mêmes figures, les mouvements morphogénétiques qui se passent exclusivement dans l'ectoblaste primaire, et en déplacent les cellules électivement par rapport à l'entoblaste, sont représentés par des flèches hachurées. Ces mouvements, qui sont comparables aux mouvements morphogénétiques des autres Vertébrés, aboutissent à la formation de la ligne primitive. Pour bien les comprendre, il faut étudier la signification de cette structure.

(3) Rauber, Balfour et van Beneden faisaient déjà de la *ligne primitive* un véritable blastopore. Gräper (1929, 1930) a repris cette conception mais a cherché à l'étayer sur des arguments assez malheureux. Pensant que pour autant qu'il ne puisse être béant comme chez un Prochordé ou un Cyclostome Pétromyzonte, le blastopore doit nécessairement contenir entre ses lèvres un bouchon vitellin, Gräper (1930) reproduit d'anciennes figures de Mammifères, ou encore des coupes postérieures de gastrulas de Reptiles, tendant à montrer qu'il existe entre les deux lèvres de la ligne primitive un "bouchon vitellin" entoblastique. Or, ces figures montrent des aspects dus à la rétraction au moment de la fixation (Mammifères), ou bien sont mal interprétées (Reptiles). Wetzel (1931) n'eut aucune peine à démontrer qu'une telle conception de la ligne primitive des Oiseaux est insoutenable, que les deux lèvres viennent au contact l'une de l'autre, se continuent directement dans la masse mésoblastique en voie d'enfouissement, sans interposition d'entoblaste. Mais pour Wetzel d'ailleurs, la ligne primitive n'est pas directement assimilable à un blastopore. Dans une première phase de la gastrulation, le "mésoblaste précoce et libre" s'invaginerait autour des lèvres primitives pour s'essaimer vers l'avant et les côtés; mais bientôt le matériel invaginé resterait stagner au niveau de la ligne pour y former un amas "indifférent", à partir duquel se différencieraient ultérieurement à la fois le système nerveux et le feuillet moyen. Reprenant donc la vieille conception du "bourgeon caudal", Wetzel assimile la ligne primitive non à un blastopore mais à un bourgeon caudal, véritable blastème contenant dans une matrice commune les matériaux de tout l'embryon.

Cette thèse est toutefois peu convaincante. Il est difficile en effet de la concilier avec la notion certaine d'induction du système nerveux par les matériaux sous-jacents; elle est en contradiction d'autre part avec les observations de Wetzel lui-même qui montre que les différents niveaux de la ligne primitive constituent respectivement des parties médianes ou latérales de l'embryon. Objection grave encore: cette conception est en quelque sorte fonction de l'imperfection de la technique. Dans l'amas cellulaire constitué par une ligne primitive, il est difficile de colorer séparément les éléments du feuillet profond invaginé et ceux du feuillet superficiel; mais celà ne peut évidemment suffire pour pouvoir affirmer que ces éléments soient confondus dans la même masse. En répétant un très grand nombre d'expériences, on peut toutefois arriver à vaincre ces difficultés et à réaliser, tout comme chez les Amphibiens, la coloration séparée de la chorde, du mésoblaste et du système nerveux (Pasteels, 1937*b*). On peut alors démontrer que tous les élé-

ments du chordomésoblaste franchissent le sillon primitif en des points définis, et que le système nerveux est formé par le matériel qui reste en surface, au-dessus de ces éléments. *Si l'on n'envisage que le point de vue cinétique, la ligne primitive est donc bien un blastopore.*

(4) Nous disposons à présent des éléments qui permettent de retrouver *les mouvements morphogénétiques* analysés chez les autres Vertébrés. Sur la fig. 11, ces mouvements qui ne concernent donc que le feuillet superficiel sont schématisés par des flèches interrompues. Ils consistent en un mouvement de *convergence* vers la ligne primitive (fig. 11 *a*, *b*, *c*, *d*) suivi d'une *invagination* au niveau de cette ligne (fig. 11 *c*, *d*). *L'extension* est tardive. Elle n'apparaît qu'au moment où la ligne primitive est à son maximum de longueur et précède de peu l'apparition de la plaque médullaire (fig. 11 $d_1$, *e*). Elle a été décrite par Gräper (1929) et Wetzel (1929) et mes observations à ce point de vue confirment celles de mes devanciers. Récemment (1938 *b*) Jacobson, mettant en doute la validité des marques colorées au blue de Nil chez les Oiseaux, croit devoir nier l'existence de cette extension progressive et prétend que le recul du "nœud de Hensen" n'est qu'apparent et résulte d'une véritable suture médiane des matériaux latéraux progressant d'avant en arrière et fermant la ligne primitive sans interposition de matériaux situés plus antérieurement. Je ferai remarquer que les résultats obtenus par des lésions du nœud de Hensen par différentes techniques (Wolff, 1936, Wetzel, 1936) montrent que son matériel est bien constitué par la chorde et, en ce qui concerne le feuillet superficiel, le plancher du système nerveux. D'autre part, des marques à l'encre de Chine, telles que les a préconisées Jacobson sans en avoir fait un usage suffisant pour emporter la conviction, m'ont montré tout récemment que cette modalité nouvelle de concrescence telle que le propose cet auteur n'est pas plus heureuse que les précédentes.[1] L'extension antéro-postérieure des matériaux qui se trouvent à l'extrémité antérieure de la ligne primitive correspond à la réalité.

(5) Le plan des ébauches ne peut, pas plus chez les Reptiles, être délimité par des marques que l'on poserait avant la gastrulation et que l'on retrouverait dans les organes primordiaux. Les marques sont trop fugaces pour cela. Mais au moment où la ligne primitive est à son maximum de longueur (fig. 11 D), on peut y poser des marques que l'on retrouvera bientôt dans les organes. Le résultat de cette prospection (Pasteels, 1937 *b*) correspond à deux réserves près aux données de Wetzel (1929). Ces réserves sont toutefois importantes. La première concerne le matériel du système nerveux. Il n'est pas confondu avec le mésoblaste mais se trouve au-dessus de lui, dans le feuillet superficiel (croissant blanc de la moitié gauche de la figure). En second lieu, la disposition du chordomésoblaste, déjà invaginé en majeure partie (moitié droite de la fig. 11 D) n'est pas non plus conforme à la description de Wetzel. S'il est exact que la chorde s'y trouve près du nœud de Hensen, les somites au niveau de la première moitié de la ligne primitive, il est erroné de dire que tout le mésoblaste latéral du corps se localiserait à la moitié postérieure de la ligne. On y trouve, en effet, du mésoblaste latéral, mais uniquement pour la partie

[1] Le détail des observations paraîtra ultérieurement.

postérieure du tronc. Celui de la moitié antérieure se trouve en dehors des somites; il s'y est placé par son invagination précoce (cf. fig. 11 C, moitié droite). On comprendra aisément comment le mouvement d'extension qui est d'autant plus marqué qu'il s'agit d'un matériel plus médian, et qui décroit dans le sens chorde parties internes des somites parties externes, viendra aboutir à la mise en place définitive des matériaux du tronc (fig. 11 E).

Pour les stades plus jeunes, il faudra tenir compte de la direction et de l'étendue des déplacements cellulaires. Connaissant la place que tient le matériel de la ligne primitive sur le blastodisque non incubé, on peut tracer à ce stade une délimitation *probable* des ébauches telle qu'on la voit sur la fig. 11 A. La connaissance des déplacements cellulaires (mouvements simultanés, invagination, convergence) nous permettra de délimiter des stades intermédiaires tels que la fig. 11 B et C. Pour ceux-ci, des marques colorées nous ont montré que leur valeur générale est bonne.

La représentation schématique de la fig. 11 A diffère à maints égards de celles que Gräper (1929) et Wetzel (1929) ont figuré pour le même stade. Sa valeur a été confirmée par des expériences d'explantations faites par Rudnick (1938). En revanche, on ne voit trop sur quels arguments est basée la version nouvelle proposée par Jacobson (1938*b*).

(6) La différence entre la gastrulation des Oiseaux et des Reptiles réside en l'apparition chez les premiers de ces mouvements simultanés des deux feuillets primaires. Ils aboutissent à un allongement précoce de l'aire embryonnaire; et en ce sens ils peuvent être considérés comme le fruit d'une simple différence de chronologie, puisque le même allongement se retrouvera chez les Reptiles après la gastrulation. A part cela, la répartition initiale et finale des ébauches est la même, les mouvements morphogénétiques sont identiques. Cependant l'allongement considérable de la région blastoporale par les mouvements simultanés va modifier du tout au tout les formes embryonnaires. Chez les Reptiles, nous avions une invagination au niveau d'un tout petit pertuis blastoporal aboutissant à un canal; chez les Oiseaux, l'invagination est étendue le long de la longue ligne primitive. En un certain sens, la ligne primitive pourrait être une gastrula de Reptile étirée de telle façon que les parois latérales du canal viendraient se placer en arrière de la voûte, et le plancher plus en arrière encore. Une fois de plus, les formes gastruléennes ne peuvent être comprises et comparées qu'en fonction de la cinétique, c'est par leur assimilation aveugle que la gastrulation des Reptiles a été si longtemps mal comprise.

## III. UN ESSAI DE SYNTHÈSE

### 1. Définition de la gastrulation

Pour qu'une synthèse soit possible, il faut que l'on connaisse bien la signification fondamentale de la gastrulation. Il importe avant tout de faire table rase de toutes les conceptions périmées. Il ne sera guère besoin de revenir sur l'*entérocœlie*, ni sur la concrescence; il a été suffisamment démontré, et pour tous les groupes, qu'il ne

s'agit là que de notions controuvées, mais d'autres devront nous retenir davantage.

En premier lieu la gastraea didermique de Haeckel. Elle ne se retrouve ni chez les Amphibiens, ni chez les Téléostéens, les Sélaciens, ni même chez les Cyclostomes; pas non plus chez les Amniotes, car le stade didermique de ceux-ci (où la chordomésoblaste se trouve encore dans l'*ectoblaste*) a une tout autre valeur que chez les Prochordés. Chez ceux-ci, on pourrait à première vue admettre qu'il existe à un certain moment l'équivalent d'une gastraea, stade à deux feuillets entourant un archentéron, communiquant avec le dehors par un blastopore. On pourrait donc supposer que le type gastraea est primitif et qu'il disparaît dans l'évolution. Mais à cette conception s'opposent les arguments suivants:

(1) Bien que la gastrula de l'Amphioxus puisse paraître comme le véritable symbole de la gastraea, les observations de Conklin nous permettent d'affirmer que le "feuillet profond" ne constitue pas une unité cohérente. Ce revêtement archentérique, malgré sa continuité apparente, est en réalité une mosaïque transitoire d'éléments disparates. Réunis par accident, parce qu'ils se sont invaginés en même temps, ils se sépareront bientôt en vertu de leurs propriétés intrinsèques.

(2) Si le type de gastrulation, telle qu'elle est réalisée dans la gastrula-gastraea avait quelque importance intrinsèque, on ne conçoit vraiment pas les raisons de sa disparition totale chez tous les Vertébrés.

C'est en effet mal poser le problème que de tâcher de définir la gastrulation par ce qu'elle aurait pu être et ce qu'elle aurait pu devenir; nous sommes là dans le marécage des hypothèses invérifiables. La gastrulation ne peut être définie que par ce qu'elle est, et par ce qu'elle est dans tous les cas. Il ne faut pas hésiter à rejeter complètement, à l'heure actuelle, l'hypothèse haeckelienne. Elle a été utile, très utile, mais elle est aujourd'hui dépassée. Elle a été imaginée dans le cadre de la "loi biogénétique fondamentale" de récapitulation. Or, telle que la concevait Haeckel, cette loi fameuse est non plus qu'une hypothèse périmée. Comme le remarque avec pertinence de Beer (1938), son noyau de vérité est une "*repetition* of ontogenetic events" chez les différentes formes. Ce n'est donc pas une loi de récapitulation mais une règle de répétition. Or, s'il est une forme embryonnaire qui ne se répète pas, c'est bien le stade à 2 feuillets entourant un archentéron.

(3) C'est mal poser le problème aussi que de définir la gastrulation par une *forme*. Or, rien n'est plus variable que les formes gastruléennes; elles paraissent même irréductibles les unes aux autres. Nous en avons donné en passant maints exemples. Et pourquoi? Il ne s'agit pas d'une forme définitive et typique comme celle du corps embryonnaire organisé mais bien d'une *transformation* toute mouvante. La gastrulation n'est que mouvements, mais ces mouvements nous les retrouvons identiques chez tous les Chordés. Eux seuls constituent la constante qui nous permettra de définir l'essence même de la gastrulation: la mise en place dans la profondeur du germe de territoires situés d'abord en superficie. Mais chez les différents Chordés, ces mêmes mouvements peuvent changer de chronologie; ils peuvent affecter des ébauches qui ont des rapports différents avec la masse vitelline, et même malgré une même disposition générale entr'elles. On comprendra aisément

pourquoi les formes gastruléennes, résultantes temporaires de ces mouvements cellulaires pourront tant différer. Et c'est le défaut des synthèses récentes comme celle de Florian (1936) et de Peter (1938*a*). Ces auteurs basent leurs comparaisons et leurs homologies sur des formes gastruléennes, ce qui ne peut mener qu'à des difficultés inextricables.

## 2. La question des "proliférations"

Si les marques colorées nous indiquent des déplacements cellulaires, n'y aurait-il pas en même temps intervention de *croissances* localisées? A priori, le fait est bien possible, puisque nous en connaissons des exemples chez les Invertébrés. Entr'autres chez les Crustacés Isopodes, c'est une croissance téloblastique qui viendra fermer définitivement le blastopore (McMurrich, 1895). Mais pour pouvoir l'affirmer, il ne suffira pas, comme on l'a fait trop souvent, de noter quelques mitoses au hasard des coupes. Une statistique rigoureuse sera une condition *nécessaire*, mais non *suffisante*. Car, pour qu'il y ait croissance, il faudra non seulement que les cellules se subdivisent plus activement, mais encore, qu'après leur division, elles augmentent de volume. Or, jamais jusqu'ici, une telle augmentation de volume n'a été recherchée, et l'école de Richards (1935) qui prétend étudier la morphogénèse par la comparaison exclusive des index mitotiques ne peut de ce fait espérer pouvoir conclure en quelque sens que ce soit. Car la simple démonstration d'une densité mitotique plus forte dans un territoire ne peut évidemment suffire à prouver que ce territoire croisse réellement, car au stade de la gastrulation, les mitoses peuvent fort bien conserver leur caractère de segmentation. On comprendra donc comment, avec une telle erreur de raisonnement, Richards & Porter (1935), Richards & Shumacher (1935), Self (1937), Bragg (1938) en arrivent à des conclusions très étranges. D'autant plus que, au point de vue de la méthode, leurs statistiques paraissent médiocres. En premier lieu, pour chaque stade un seul embryon a été utilisé, alors qu'il aurait fallu faire des numérations sur plusieurs embryons du même stade, pour juger de la constance éventuelle des résultats. Ensuite, ces auteurs ont omis de calculer la marge des erreurs statistiques. Or, cette omission est d'autant plus grosse que les index mitotiques (c'est-à-dire le pourcentage de cellules en division) dont la comparaison sert de base au raisonnement sont souvent très voisins. Prenons par exemple un tableau de la page 405 du mémoire de Richards & Shumacher (1935):

| Summary for 5-day embryo | Total cells | Active division | Mitotic rate |
|---|---|---|---|
| Ectoderm of embryonic shield | 3191 | 19 | 0·59 |
| Ectoderm forming neural groove | 183 | 0 | 0·00 |
| Primary entoderm of embryonic shield | 484 | 1 | 0·207 |
| Ectoderm of germ ring | 1001 | 5 | 0·49 |
| Primary entoderm of germ ring | 339 | 1 | 0·27 |

Si nous calculons la marge d'erreurs d'après la formule classique $p \pm 3\sqrt{\frac{p(1-p)}{N}}$ où $N$ représente le nombre total de cellules et $p$ le rapport $\frac{\text{nombre de mitoses}}{N}$, nous arriverons aux résultats suivants:

| | |
|---|---|
| 0·59 | ±0·36 |
| 0 | ? |
| 0·207 | ±0·63 |
| 0·49 | ±0·66 |
| 0·27 | ±0·84 |

Nous nous dispenserons de commentaires.

Récemment, Preto a dénombré soigneusement toutes les mitoses de deux embryons de Rat pris à des stades différents de la ligne primitive. Les conclusions de ce mémoire sont plus prudentes et en accord avec les *faits* décelés par la méthode des marques colorées. Deux ordres de résultats doivent être considérés dans ce mémoire.

(*a*) Il existe entre les trois feuillets de très fortes différences d'index mitotique (ectoblaste: 85·3; mésoblaste: 39·6; entoblaste: 12·8). Ces chiffres très différents proviennent d'une numération d'un très grand nombre de cellules et sont donc statistiquement irréprochables: l'auteur est dès lors en droit de conclure que l'activité mitotique est dès ce stade différente dans les trois feuillets.

(*b*) L'auteur a voulu également tâcher de calculer dans chacun des feuillets l'index mitotique de tranches plus ou moins antérieures ou plus ou moins latérales par rapport à la ligne primitive. Malheureusement, les chiffres deviennent forcément bas et la marge des erreurs que Preto a malheureusement omis de calculer devient telle que toute conclusion peut être considérée comme superflue.

Pour ma part, profitant de ce que chez la Truite les circonstances (taille uniforme des cellules, densité constante de celles-ci, absence de vitellus) semblaient exceptionnellement favorables, j'ai eu recours à un procédé plus sommaire: il consistait à dessiner toutes les coupes d'un embryon à la chambre claire et à y pointer les mitoses, à mesurer la densité mitotique par unité de volume. Durant toute la gastrulation, cette densité est apparue constante pour un même embryon, sauf en ce qui concerne l'ectoblaste extra-embryonnaire, où elle devenait plus faible. J'en avait déduit, puisque par ailleurs les cellules gardent une taille uniforme, que la gastrulation s'y déroule exclusivement sous l'influence des déplacements cellulaires et sans l'intervention de proliférations localisées (Pasteels, 1936*b*). J'ajouterai à présent que cette conclusion est peut-être trop rigide et que ce ne sont guère que de grosses proliférations dont j'ai pu exclure à coup sûr l'existence par la méthode employée.

Quoiqu'il en soit, *on n'a pu jusqu'ici démontrer de façon certaine qu'une prolifération éventuelle joue un rôle dans la gastrulation des Vertébrés*; on n'a pu encore moins prouver l'assertion tant de fois répétée, et encore tout récemment par Holmdahl (1939) qu'il existe une prolifération au niveau de la lèvre blastoporale.

Pour Holmdahl (1925 à 1935, 1939) il existe deux morphogénèses différentes.

L'une primaire (aussi appelée indirecte par Peter, 1934*a*) concerne la partie antérieure du tronc. Un blastème, la lèvre blastoporale, constitue des feuillets, à partir desquels s'édifient les organes. Quant à la partie postérieure du corps, elle est constituée "directement" (Peter) à partir du blastème qu'est le bourgeon tronco-caudal, sans passer par l'intermédiaire des feuillets. Il y a beaucoup à redire à ce propos. La lèvre blastoporale n'est pas un blastème mais un simple point de réflexion des cellules superficielles dans la profondeur. Quant au bourgeon tronco-caudal, son aspect "indifférent" cache une hétérogénéïté fondamentale. Le caractère dense des cellules à allure "embryonnaire" est dû au fait que les territoires postérieurs se différencient plus tard et constitueront longtemps une sorte de phase gastruléenne dans un embryon dont les parties antérieures sont déjà bien différenciées. Par ailleurs, il apparaît bien que la croissance est à un certain moment plus intense en arrière de l'embryon que dans ses parties antérieures. Mais il n'y a là que des différences quantitatives dont on aperçoit la gamme des transitions et qui ne permettent pas la distinction entre deux morphogénèses qualitativement différentes, bien tranchées à un certain niveau du corps comme le prétendent Holmdahl et Peter. On ne saurait trop se défier de concepts rappelant les céphalo- et urogénèses bien caduques à présent. *La gastrulation et la morphogénèse sont en réalité unitaires* puisque l'analyse permet de déceler les *mêmes* mouvements morphogénétiques dans la mise en place des organes tout le long de l'embryon.

### 3. Deux formes gastruléennes constantes

Une forme embryonnaire qui semble bien constante et qui joue un rôle énorme dans toutes les disciplines morphologiques, voire pathologiques, c'est *le feuillet*. Encore faut-il prendre garde de n'appliquer la notion de feuillet que pour l'embryon *tridermique*: postgastruléen et non pas dans une phase didermique transitoire. Car il est évident qu'un "ectoblaste" de l'embryon didermique d'Amphioxus ne peut être homologué au "feuillet"[1] superficiel de l'embryon didermique des Amniotes; celui-ci comprend en effet à la fois l'ectoblaste et le chordomésoblaste (et peut-être une partie de l'entoblaste). D'autre part, le terme même de feuillet ne doit pas être pris dans un sens trop rigoureux puisqu'il est absolument indifférent que des organes se soient constitués par des feuillets (partie antérieure du tronc) ou par un bourgeon massif, continu, non délaminé (partie postérieure du tronc). Le feuillet n'est donc pas le tabou que vénérait l'ancienne embryologie, ce n'est qu'un rang cellulaire. Des organes ne présenteront nullement, comme on le croyait, des affinités *parce qu*'ils proviennent du même feuillet mais au contraire ils proviennent du même feuillet parce qu'ils proviennent de territoires voisins et ont suivi un chemin commun. C'est en ce sens que la "spécificité des feuillets" gardera une certaine valeur malgré des exceptions apparentes telles que la formation de cartilages à partir de crêtes ganglionnaires (travaux de Stone, Knouff, Raven, etc: références dans le *Traité d'Embryologie* de A. Brachet, 2ème édition, 1935).

Une autre forme gastruléenne constante c'est le blastopore; son sens primitif

[1] Ici, les termes d'ecto- et d'entophylle proposés par Celestino da Costa, n'ayant qu'une valeur topographique et non génétique trouveront leur emploi.

signifie "bouche" ("Urmund" en allemand). S'appuyant sur cette signification littérale, on serait en droit, avec Wetzel, de nier l'existence d'un blastopore quand il n'y a pas d'archentéron. Mais pourquoi priver, dans certains cas, comme chez les Oiseaux, la gastrulation d'un de ses attributs essentiels? On l'avait mal définie. Oublions le sens original du mot et insistons sur le sens *fonctionnel* du blastopore: *l'orifice d'entrée des cellules en voie d'invagination.* Un tel blastopore se retrouve partout et toujours.

### 4. Variantes de la gastrulation chez les Chordés et leurs origines éventuelles

Pour connaître le mécanisme de l'évolution de la gastrulation chez les Chordés, il faudrait connaître le mécanisme et la source des mouvements morphogénétiques. Des travaux expérimentaux récents qui ne pourront qu'être envisagés brièvement ici mais dont on trouvera mention plus complète ailleurs (cf. Dalcq, 1938*b*) nous fournissent quelques lueurs à ce sujet. En soumettant des œufs d'Amphibiens à un retournement forcé tel que le vitellus, déplacé par la pesanteur, vienne adhérer à des points divers de la surface ovulaire, on peut suivre l'apparition des lèvres blastoporales aux points les plus divers. Chaque blastopore traduit une constellation complète de mouvements morphogénétiques d'une gastrulation. Suivant les cas, on en trouvera un, deux, même trois dans le même œuf. Il est évident qu'ils ont été constitués *de novo* par la modification de l'œuf apportée par l'expérience; il est évident aussi que les facteurs mis en jeu ne sont rien d'autre que ceux qui président au développement normal. L'analyse des cas a pu montrer que ces facteurs sont au nombre de deux (sans compter un génome normal): le gradient vitellin et un champ cortical ayant son maximum d'intensité du côté dorsal (Pasteels, 1938). Le champ cortical est rigide et stable; le vitellus, déplacé par l'expérience. Or, la gastrulation débutera toujours au point de la marge d'un amas de vitellus dense qui est situé le plus près du centre du champ. Partant de ces données et d'autres récoltées par Dalcq par l'expérimentation sur les œufs d'Ascidies et d'Amphibiens, nous avons envisagé dans deux mémoires communs "une conception nouvelle des bases physiologiques de la morphogénèse" (Dalcq & Pasteels, 1937, 1938).

En ce qui concerne la gastrulation, l'invagination serait déclanchée dès qu'un taux liminaire de produits résultant de l'interaction du gradient et du champ serait atteint. Au sein de la ceinture marginale, la ségrégation en chorde, somites, lames latérales dépendrait du taux relatif des deux facteurs $C$ (cortex) et $V$ (vitellus) engagés dans la combinaison; or, on peut poser que les mouvements de convergence et d'extension sont liés aux prédispositions chordales et mésoblastiques. À l'aide de ces données, nous avons pu reproduire graphiquement un plan d'ébauches ressemblant dans ses lignes générales à celui des Amphibiens (Dalcq & Pasteels, 1938, pp. 269 et 272).

Un coup d'œil récapitulatif sur la gastrulation des Chordés nous amènera à considérer d'abord que la particularité des Prochordés consiste en la subdivision de la zone marginale en deux parties séparées: une ventrale, mésoblastique et une dorsale, chordale. L'organisation de l'œuf d'*Ascidiella* a été étudiée par Dalcq

(1938*a*) par des expériences de mérogonie double de l'œuf vierge. Il en résulte qu'il faut considérer chez cette espèce—et vraisemblablement chez tous les Prochordés—l'existence outre le champ cortical et le gradient vitellin communs à tous les Chordés, d'un mésoplasme intervenant dans la constitution du croissant mésoblastique ventral qui n'est pas, comme nous l'avons vu, l'équivalent du mésoblaste des Vertébrés.

La caractéristique de tous les Vertébrés non Amniotes, c'est l'existence d'une ceinture chordomésoblastique marginale continue séparant l'ectoblaste de l'ento-

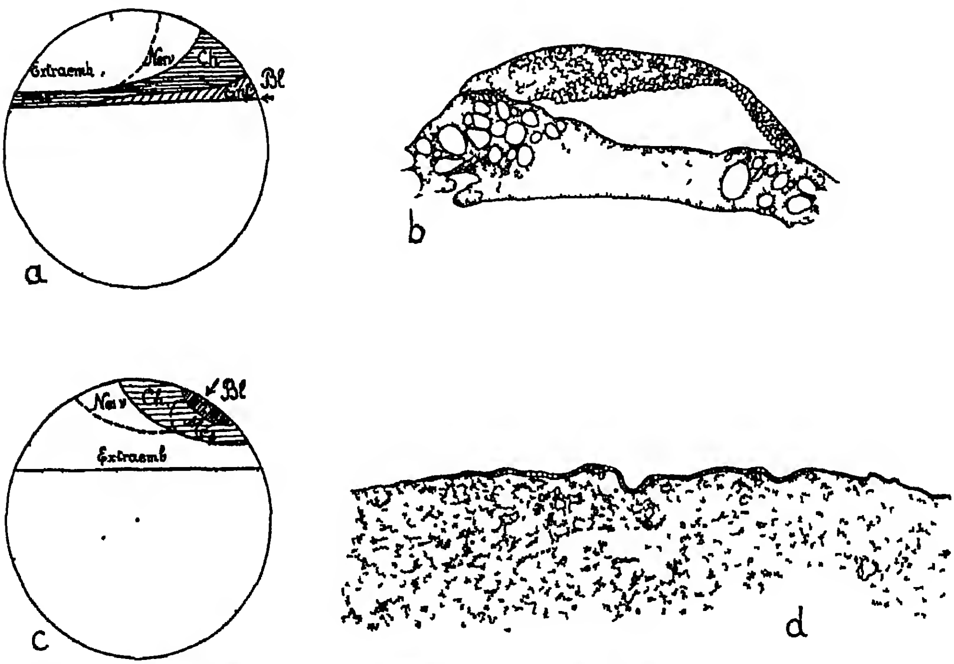


Fig. 12. (*a*) Schématisation d'un œuf télolécithique d'Anamniote. Œuf entier vu de côté. En ponctuation: vitellus; *ent.* entoblaste, *ch.* chorde; *més* mésoblaste; *nerv.* neurectoblaste; *extraembr* épiblaste extra-embryonnaire; *Bl.* blastopore (*b*) Coupe sagittale d'une blastula de Truite (une partie du vitellus seulement est représentee). (*c*) Schématisation d'un œuf télolécithique d'Amniote. Œuf entier vu de côté Mêmes symboles que pour (*a*) (*d*) Coupe sagittale d'une blastula de *Clemmys leprosa* (une partie seulement du vitellus est représentée).

blaste. Cette règle ne souffre d'exception que chez certains Sélaciens, tel que *Scyllium canicula*, chez lesquels vraisemblablement par suite de la forte accumulation vitelline, cette zone est interrompue du côté ventral. Lorsque chez ces Vertébrés non Amniotes la segmentation est partielle, on y observe en somme un plan d'Amphibien, dont toute la zone vitelline sous-blastoporale resterait insegmentée; la fig. 12*a* nous montre une blastula de Téléostéen coiffant sa masse vitelline et vue de profil: le plan est celui de la fig. 6*a*, sauf que l'entoblaste qui cache en surface une partie de la zone marginale y a été représenté; l'épiblaste extra-embryonnaire a été délimité également. Notons ces deux points essentiels: (*a*) *le blastopore est à la limite qui sépare le blastodisque de la masse vitelline*; (*b*) *l'ectoblaste extra-embryonnaire est intradiscal, sans rapports directs avec le vitellus.*

En ce qui concerne les mouvements morphogénétiques, nous constatons une curieuse modification de chronologie qui apparaît dès les formes les plus inférieures pour s'accentuer par la suite au cours de l'évolution. En effet, chez les Pétromyzontes, l'invagination blastoporale progresse très lentement dans le sens dorso-ventral; les matériaux dorsaux ont pu s'invaginer depuis longtemps et ont pu déjà commencer à s'enfouir en profondeur. Ce retard est déjà moins accentué chez les Téléostéens; et beaucoup moins encore chez les Amphibiens. Enfin dès les Reptiles, chordomésoblaste dorsal et mésoblaste ventral débutent leur invagination en même temps.

Remarquons qu'à part ces différences de chronologie, et à part aussi la quantité plus ou moins grande de vitellus qui s'accumule au pôle végétatif, la gastrulation ne subit que peu de variations chez les Vertébrés non Amniotes. Il est alors caractéristique de voir qu'il existe à l'intérieur même de la classe, probablement polyphylétique (cf. Säve-Soderbergh, 1934) des Amphibiens, des différences entre la gastrulation des Urodèles et des Anoures. Ces différences tiennent à la situation de la lèvre blastoporale par rapport à l'entoblaste et au mésoblaste; à la séparation plus ou moins précoce de ces deux feuillets; à la différence de chronologie de l'invagination mésoblastique; à la différence de hauteur de la zone marginale. Tandis que l'œuf des Téléostéens pourrait être en somme un œuf d'Amphibien dont la masse entoblastique sous-blastoporale serait insegmentée, ce que nous savons de la gastrulation des Dipneustes les rapproche davantage des Urodèles.

Si nous considérons à présent la gastrulation des Amniotes, nous constatons un saut brusque dans l'évolution. En effet, si nous comparons sur la fig. 12 au schéma de l'œuf de Téléostéen que nous avons vu plus haut avec un schéma semblable d'un œuf de Reptile (dont la masse vitelline est supposée toutefois considérablement réduite) nous apercevrons (fig. 12*c*) un aspect tout différent. La répartition générale des ébauches entr'elles est la même, mais chez les Reptiles l'anneau chordo-mésoblastique entoure un *blastopore intradiscal*; en revanche, c'est l'ectoblaste extra-embryonnaire qui se trouve à la limite du vitellus. Or, nous avons vu le contraire chez les Amniotes. Quel est l'origine de ce chassé-croisé? Nous avons vu que la contact du vitellus était un facteur décisif pour l'apparition du blastopore et des mouvements gastruléens. Or, il est à noter que les rapports entre blastodisque et vitellus sont très différents chez les Anamniotes méroblastiques et chez les Amniotes. Une coupe sagittale d'une blastula de Téléostéen telle qu'on la voit sur la fig. 12*b* montre strictement l'équivalent d'une blastula d'Amphibien mais dont toute la partie sous-blastocœlienne serait insegmentée. La large cavité séparant le blastodisque du vitellus s'est creusée au cours de la segmentation, elle est nettement séparée du vitellus sous-jacent par une membrane, c'est un blastocœle au sens strict du mot. Le blastodisque ne touche au vitellus que par sa marge qui entoure la base du blastocœle. On conçoit facilement que le blastopore, se constituant au contact du vitellus, ne puisse être que marginal. Or, une même coupe de la Tortue *Clemmys leprosa* (fig. 12*d*) nous montrera un tout autre aspect. Le blastodisque s'est étalé au contact même du vitellus. Nous sommes à la veille de la gastrulation et nous ne voyons pas de blastocœle. S'il en existe un chez d'autres Amniotes, ce

ne pourrait être que la fente séparant l'ectophylle et l'entophylle du stade didermique. Quoiqu'on en ait dit à ce sujet (cf. Peter, 1938*a*), *la cavité sous-germinale des Sauropsides n'est pas l'homologue d'un blastocœle*. Le blastocœle est strictement une cavité intercellulaire, apparaissant précocement au cours de la segmentation et limitée de toutes parts, y compris du côté du vitellus chez les Amniotes télolécithiques. Chez ceux-ci, la masse vitelline est englobée dans une membrane incomplète et représente en quelque sorte une vaste cellule. Le blastocœle contient un liquide distinct du vitellus. Or, la "cavité" sous-germinale des Sauropsides n'est en somme une "cavité" que sur coupes microscopiques. Tout embryologiste familier de l'œuf d'Oiseau vivant sait qu'elle est remplie de vitellus liquifié en continuité directe avec le vitellus sous-jacent et que, contrairement à ce qui se passe chez un Poisson, il est impossible d'enlever le blastodisque sans que le jaune ne s'écoule. Cette "cavité" ne se forme en réalité que plus ou moins tard (très tard chez la Tortue, au moment même de la gastrulation) et résulte de la digestion progressive du vitellus par les cellules sous-jacentes. Il en résulte que, chez les Sauropsides, l'ensemble du germe est toujours en contact immédiat avec le vitellus ou ses produits de digestion. On conçoit donc que, contrairement à ce qui se passe chez les Téléostéens, le blastopore *puisse* être intradiscal. Pourquoi il l'est réellement est toutefois un autre problème dont la solution n'apparaît pas encore.

Quoiqu'il en soit, cette dissociation de l'englobement du vitellus et de la gastrulation mène à deux conséquences importantes. La première est que l'entoblaste est en quelque sorte détaché du vitellus. Sans doute faut-il voir la raison de son invagination particulièrement précoce chez les Reptiles, peut-être aussi la raison de sa constitution au moins partielle par délamination chez les Oiseaux quoiqu'il vaudrait mieux s'abstenir de se prononcer avant d'avoir recueilli de nouveaux résultats. La seconde conséquence c'est que la masse vitelline qui était englobée pendant la gastrulation et par le blastopore chez les Téléostéens, est chez les Reptiles, entourée par l'ectoblaste extra-embryonnaire, indépendamment de la gastrulation. Cette épibolie extra-embryonnaire peut se faire avant (Caméléon: Peter, 1934*b*), pendant ou après gastrulation. Dalcq (1937, 1938*b*) a très justement noté ses rapports avec la formation de l'amnios, dont le stade d'apparition est aussi si variable mais toujours contemporain de l'épibolie.

L'invagination précoce de l'entoblaste chez les Reptiles a pour résultat une modification importante de la cinétique. L'anneau marginal entoure en effet un petit pertuis blastoporal et on conçoit facilement comment une invagination concentrique vienne y constituer un canal qui s'immisce entre les deux feuillets. Nous retrouvons à peu près la disposition caractéristique de *Lampetra* avec une différence fondamentale toutefois: chez le Cyclostome ce canal est un archentéron véritable, le plancher étant entoblastique, tandis que chez le Reptile ce plancher est mésoblastique. C'est par la perforation progressive de ce plancher par le mouvement de divergence que nous avons analysé plus haut que chez le Reptile se rétablit la disposition typique d'une voûte archentérique.

Une caractéristique aussi de la cinématique des Amniotes, c'est l'apparition tardive—surtout chez les Oiseaux—des mouvements d'extension. Toutefois,

Vandebroek l'a signalé aussi chez *Scyllium*. Cela est dû vraisemblablement au fait que la progression antéro-postérieure de la morphogénèse y est très lente.

Reste à expliquer l'apparition de la ligne primitive des Homéothermes. Elle est due évidemment à l'élongation de la zone blastoporale sous l'effet des mouvements simultanés qui déforment de la même façon les deux feuillets primaires. Ces mouvements ont manifestement pour *effet* une élongation précoce de toute l'aire embryonnaire; mais leur *cause* reste mystérieuse.

Il serait enfin intéressant, quoiqu'il ne faille pas s'attendre à des surprises bien vives de ce côté, de pouvoir étudier la cinétique des formes de transition entre gastrulation reptilienne et ligne primitive, telles qu'on en rencontre chez certains Mammifères.

Nous voyons donc que pour progresser, l'étude de la gastrulation devra se poursuivre sur deux plans connexes s'éclairant l'un l'autre: le point de vue descriptif, déjà largement déblayé et la point de vue causal, analytique sur lequel de premières recherches entr'ouvrent de vastes perspectives.

## IV. RÉSUMÉ

A. (1) La gastrulation des Amphibiens est décrite en détail d'après les recherches de Vogt (1929*a*) et de ses continuateurs. Sont envisagés successivement, le plan des ébauches de la jeune gastrula, les mouvements cellulaires d'ensemble dits "mouvements morphogénétiques". Ceux-ci consistent en invagination, convergence, extension, épibolie. Enfin, sont résumées les conclusions nouvelles apportées par Vogt: la correspondance éventuelle des axes ovulaire et embryonnaire, l'inexistence de la concrescence, le manque de pertinence de la théorie cœlomique, l'origine de la chorde, la valeur limitée de la théorie de la gastraea, la réfutation de la subdivision du développement en céphalo-notogénèse, etc.

(2 et 3) Chez les Téléostéens et les Sélaciens malgré l'existence d'une masse vitelline insegmentée le plan des ébauches et les mouvements morphogénétiques présentent la plus grande analogie avec ceux des Amphibiens. En ce qui concerne les premiers, il convient d'insister sur la similitude de la cinétique opposée à la grande différence des formes gastruléennes.

(4) Une version nouvelle de la gastrulation du Cyclostome *Lampetra* est décrite; ce qui permet d'affirmer qu'il n'y a, entre cette gastrulation et celle des Amphibiens, d'autre différence que chronologique; contrairement à ce qu'affirment Weissenberg, la gastrula de *Lampetra* n'est pas didermique mais bien tridermique.

(5) La gastrulation de l'Amphioxus et de l'Ascidie est résumée d'après les travaux de Conklin (1932) et de Vandebroek (1939). La grande similitude de la dynamique gastruléenne avec celle des Vertébrés est soulignée; le stade didermique et l'entérocœlie ultérieure ne sont considérés que comme des aspects purement contingents, le "feuillet" profond étant en réalité un complexe; le croissant mésoblastique ventral n'est pas l'homologue du mésoblaste des Vertébrés.

(6) Chez les Reptiles, nous aboutissons aux conclusions suivantes: (*a*) les mouvements morphogénétiques sont semblables à ceux des Anamniotes, mais disposés dans un autre ordre chronologique; (*b*) l'"entérocœlie" ne correspond qu'à

une mauvaise interprétation des coupes microscopiques; (*c*) le canal chordomésoblastique n'est pas un archentéron véritable; (*d*) il n'y existe pas de "plaque primitive" analogue à la ligne primitive des Oiseaux; (*e*) le plan des ébauches se distingue par la position "intradiscale" du blastopore, complètement séparé de la masse vitelline; (*f*) il y a dissociation complète entre la gastrulation proprement dite et l'épibolie de l'épiblaste extra-embryonnaire.

(7) Chez les Oiseaux et Mammifères, (*a*) la question difficile et controversée de la formation et de la signification de l'embryon didermique est discutée, (*b*) sont décrits les "mouvements simultanés" des deux feuillets primaires, apparition nouvelle dont dépend l'élongation de la zone blastoporale, (*c*) la thèse faisant de la ligne primitive un blastopore au sens cinétique du mot est défendue, (*d*) les mouvements morphogénétiques sont décrits en accord avec ceux des autres Vertébrés, (*e*) le plan des ébauches est figuré de la même façon que celui des Reptiles.

B. (1) La gastraea didermique de Haeckel n'a plus qu'un intérêt historique; on ne peut définir la gastrulation par des formes; les formes gastruléennes sont contingentes et dépendent de la chronologie variable des mouvements morphogénétiques; ceux-ci seuls peuvent nous définir la gastrulation qui est une cinétique.

(2) L'intervention éventuelle de "proliférations" dans le mécanisme de la gastrulation est discutée: aucune preuve positive n'en a été donnée jusqu'ici; contrairement à la thèse de Holmdahl la morphogénèse des Vertébrés doit être considérée comme unitaire.

(3) Deux formes gastruléennes sont constantes, mais il importe d'insister sur leur signification réelle. Le *feuillet* n'est pas un organe primitif, mais un rang de cellules suivant le même chemin; le *blastopore* n'est pas une "bouche primitive" mais le point d'entrée des cellules à l'intérieur du germe.

(4) Les variations de la gastrulation chez les Chordés sont passées en revue en tâchant, autant qu'il se peut, d'en donner un aperçu causal.

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## ADDENDUM

Au dernier congrès (1939) de l'Anatomische Gesellschaft, Vogt (*Anat. Anz.* **88**, Erg.heft, p. 112) a repris sa thèse sur l'origine des matériaux caudaux des Urodèles au stade de la plaque médullaire. Les ébauches somitiques seraient localisées uniquement au niveau des bourrelets médullaires, tandis que le fond de la plaque, au contact de la lèvre dorsale de la fente blastoporale, serait occupé par du matériel chordal. Vogt combat ainsi énergiquement les conclusions de Bijtel et de Nakamura, qui vouent intégralement aux matériaux somitiques la partie postérieure de la plaque neurale. Des marques de contrôle (Axolotl et Pleurodèle), faites ces dernières semaines, me permettent un avis catégorique: la thèse de Bijtel et de Nakamura est exacte, celle de Vogt erronée (sous presse: *Bull. Acad. Belg. Cl. Sci.*).

# HEARING IN INSECTS

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(*Received* 5 *May* 1939)

CONTENTS



## I. INTRODUCTION

The study of hearing in insects has a long and honourable history. Johannes Müller (1826) was the first of a succession of scientists famous in other fields who have been attracted by the problem. Nevertheless, two causes have operated to prevent a scientific analysis. The first is technical. Although sound as a stimulus is theoretically capable of the most precise adjustment as to intensity, frequency, wave form and direction, it is only in the last decade that such adjustment has become possible in practice, and the production of sound stimuli of known characteristics is still a matter of considerable difficulty and expense. Moreover, since this technical advance has been due almost entirely to the development of wireless telephony and of the speaking film, it has been directed towards air-borne sounds within the compass of the human ear, and the production of controlled sounds outside this range or in the heavier medium of water is exceedingly difficult and uncertain. In the earlier work on hearing, therefore, little attempt to control the intensity or the harmonic content of sound stimuli could be made. It is consequently impossible in most cases for a reviewer to assign the proper weight to pieces of conflicting evidence which have been brought forward from time to time.

The second cause is metaphysical, and centres round the meaning to be attached to the word *hearing*. It cannot be denied that many of the naturalists of the last century applied terms derived from human experience to the responses of animals with the greatest freedom and without considering their implications. A reaction

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followed, largely influenced by Loeb and von Uexküll, tending towards an entirely mechanistic outlook and culminating in the proposal of Beer *et al.* (1899) to eliminate from the study of invertebrate behaviour all reference to human experience. They proposed to substitute new terms, e.g. phonoreception for hearing, photoreception for vision, etc. This proposal largely failed of its object. On the one hand, it was ignored by the majority of naturalists and morphologists. On the other, where it was adopted by the experimentalists, they often took over the new terms, but used them merely as synonyms for the old. The language was new, but the meaning was the same, and the core of the problem remained untouched. This problem, restated with reference to the subject of this review, is briefly the problem of defining the nature of the evidence which will justify the attribution of a sense of hearing to animals which differ radically from man in their organization and into whose "minds", if such exist, we have in the nature of things no opportunity of seeing. Quite clearly the substitution of the term "phonoreception" for hearing evades the real issue. For phonoreception, if it means anything, means sensitivity to sound or responsiveness to sound; and sound unqualified means a class of stimuli which produce the sensation of hearing in normal human beings. The use of the term phonoreception, therefore, at most implies that we have no means of estimating the quality of the sensory experience of lower animals and therefore no justification in assuming that it has anything in common with the human experience of hearing. It has in no way cleared up the muddle which inevitably arises when a human observer endeavours to distinguish between phonic and tactile senses in an animal on the basis of his own sensory experience. This confusion is apparent right through the literature up to the present time. Von Buddenbrock (1937) distinguishes between a "sensitivity to vibrations" mediated by tactile end-organs and "*der echte Gehörsinn*" and defines the latter as follows: "Wir werden einen Gehörsinn nur dann als bewiesen ansehen, wenn sich nachweisen lässt, dass das betreffende Sinnesorgan seiner Struktur nach spezifisch auf die Wahrnehmung von Schallwellen eingestellt ist. Das Kriterium des Gehörsinns ist daher das besonders ausgebildete Gehörorgan, welches als characterisches Element in stets wiederkehrende Weise eine oder mehrere *Membranen* besitzt, die zum Mitschwingen durch *Resonanz* befähigt sind." The italics are mine. Such a definition is clearly founded on the human analogy. The first sentence seems to imply that there is some fundamental distinction between sound waves and other mechanical stimuli, presumably on the grounds that touch and hearing are distinct in man, for the assumption can hardly be maintained in the face of the evidence bearing on the evolution of sound receptors either in vertebrate or other animals. But the second sentence goes even further. The final criterion of an auditory organ is not its specific sensitivity to sound waves, but a resemblance in certain arbitrary structural particulars to the human ear. If this criterion be accepted, fish are deaf by definition (but cf. von Frisch, 1936[1]), and only those insects with tympanic organs can hear. It is then necessary to invent another sense, sensitivity to vibrations or *Erschütterungssinn* to explain the responsiveness to sound of animals which have no *echter Gehörsinn*. This invention has had unfortunate consequences, because of

[1] *Biological Reviews.*

the tendency to regard sensitivity to vibrations as a special kind of touch sense in quite a separate category from true hearing instead of regarding it as including true hearing, which would be both logical and consistent with what is known of the evolution of hearing organs.

In this review I shall adopt the following definitions. An insect will be said to hear when it is demonstrably responsive to sound. An auditory organ is one which can be shown to mediate the above response or which can itself be shown by more direct means to respond to sound. Sound is arbitrarily defined as consisting of disturbances of the *air* of low intensity irrespective of whether or not they are of such a frequency and intensity as to produce the sensation of hearing in a human being. This definition of sound is open to objections. First, it excludes water-borne sounds. This is at the present moment immaterial, since our information about the responses of insects in water to sound is practically nil, and what little there is is inconclusive. Secondly, it excludes sounds transmitted through the substrate. Below I shall refer briefly to the evidence for the sensitivity of insects to sounds of this kind, though it is not strictly within the province of this review. Thirdly, the only distinction made between hearing and the tactile senses is based on the intensity factor and is quite arbitrary. At the present time no confusion arises in practice because the sensitivity of the auditory organs so far investigated is of a different order from the sensitivity of end-organs usually regarded as tactile. But the distinction is not fundamental, and it can be regarded as certain that further work will demonstrate the existence of end-organs intermediate in sensitivity between "hearing" and "tactile" end-organs. There will then be no justification for attempting a sharp separation.

Historically scientific interest focused first on the tympanic organs of the Orthoptera. Müller (1826) described the structure of the tympanic organ of an acridiid and assigned an auditory function to it on morphological grounds, though he provided no experimental evidence. Von Siebold (1844) first described the tympanic organs of tettigoniids and gryllids as auditory organs, also without experimental confirmation. Johnston (1855) showed that chordotonal[1] organs were not only found in association with tympanic membranes but also occurred in the second antennal joint of insects of different Orders, reaching a relatively very large size and a considerable elaboration of structure in the antennae of culicids. These observations led him to ascribe an auditory function to the antennae, a view which later received support from Mayer (1874) who showed that the long hairs on the antennae of male culicids

[1] The words "organ" and "chordotonal" need explanation. The terminology of insect sensory structures was elaborated before there was experimental evidence as to the function of the majority of such structures. In English the term sensillum (*pl.* sensilla) or alternatively sensilla (*pl.* sensillae) is used to denote the equivalent in insects of a *sensory end-organ* in vertebrates, viz. the primary sense cell and functionally associated structures. In German and sometimes in English the word *organ* is applied to a single sensillum, e.g. campaniform sensillum = *kuppelförmiges Organ*. The common English usage is, however, to apply the term organ only to groups of similar sensilla and I have adopted this practice.

The word *chordotonal* derives from the early belief, which is certainly untenable, that the sensilla associated with tympanic organs were resonant elements like stretched strings. Nowadays it is customary to apply the adjective *chordotonal* (German *stiftführend*) to all sensilla which present a similar histological picture to those associated with tympanic organs. This practice, though logically indefensible, has been hallowed by custom, and will be adopted here.

were resonant to a tone of the same pitch as the hum of the female in flight, and concluded that their vibration might be transmitted to Johnston's organ. A number of earlier observations (Kirby & Spence, 1828; Newport, 1836; Hicks, 1859; Will, 1885) had been taken to indicate an auditory function for the antennae on the ground that many insects of diverse orders responded to sounds by moving them. This was clearly of little evidential value. Many mammals respond to sound by reflex movements of the eyes. Lehmann (quoted by Lubbock 1888, Ch. v), moreover, had shown long before that crickets remained as responsive to sound after removal of the antennae as before. But Rudow (1870) still regarded the antennae as the primary auditory organs on the grounds that responsive movements could be obtained by sound stimulation of the head of a decapitated cricket. Moreover, experimental evidence of the responsiveness of insects not possessed of tympanic organs was accumulating. It was already clear to Lubbock (1888) that the conflict could only be resolved if neither tympanic organs nor antennae were the sole auditory organs. Meanwhile the work of Leydig, Hicks, Graber, Burger and F. Müller had demonstrated that chordotonal organs were present in all the principal orders of insects (see Graber, 1882*a*) both in early larvae and adults. (Tympanic organs are found only in adults and late nymphal instars.) It began to seem impossible that the function of all chordotonal sensilla was responsiveness to sound, just as it also seemed impossible to attribute all responses to sound to chordotonal sensilla. This then was the position up till the first decade of this century. Responses to sound had been demonstrated in a wide variety of insects. Organs which might be responsive to sound had also been described in a much smaller number. But no careful experimental work had been done to link the morphological knowledge with the many scattered observations of behaviour.[1] The advances which have been made since that period are in a large measure due to the exemplary experiments of Regen and Minnich to which detailed reference will be made below. It is with the experimental evidence that this review will be chiefly concerned. For structural details the reader is referred particularly to the comprehensive monographs of Schwabe (1906), Sihler (1924), Snodgrass (1926) and Eggers (1928).

In order that the experimental evidence reviewed below shall be comprehensible, it is necessary to include here a very brief and non-technical disquisition on the physics of sound detection. In a homogeneous medium a local disturbance such as might be caused by the expansion of a small volume of the medium will produce both a local increase in *pressure* and a *displacement* of contiguous particles away from the source of the disturbance. In a medium with finite density and compressibility such a disturbance will be transmitted as a spherical wave, so that at some later time more distant regions of the medium will experience both a pressure increase and a displacement. In a fluid and non-rigid medium such as air or water the velocity of transmission of the wave is determined solely by the density and compressibility of

[1] Graber (1882*b*) had demonstrated the responsiveness of a cockroach (*Blatta germanica*) to sound. The responsiveness was here undoubtedly mediated by the anal cerci (see below) and not, as Graber believed, by chordotonal sensilla. His experiments on *Corixa* were not confirmed by Hagemann (1910).

the medium and the displacement is radial and normal to the wave front. The magnitudes of pressure change and of displacement are inversely proportional to the distance from the source. It is important to realize that the above statements are true not only for periodic disturbances of such a frequency that, humanly speaking, they are termed sound waves, but of any type of disturbance whatever, whether periodic or not, with the proviso that the proportionality law does not hold in the neighbourhood of the source for large and rapid displacements.[1]

When the disturbances are periodic and sinusoidal, it can be shown theoretically that the pressure amplitude ($p$) and the displacement amplitude ($\xi$) at any point distant from the source are connected by the following expression:

$$\frac{p^2}{\rho c}=\frac{\omega^2\xi^2\rho c}{2}=I,$$

where $\omega$ = angular frequency (i.e. frequency in cycles per second $\times 2\pi$), $\rho$ = density of the medium, $c$ is the velocity of propagation of waves in the medium, and $I$ = intensity of the wave train, defined as the power transmitted across unit area normal to the wave front at that point.

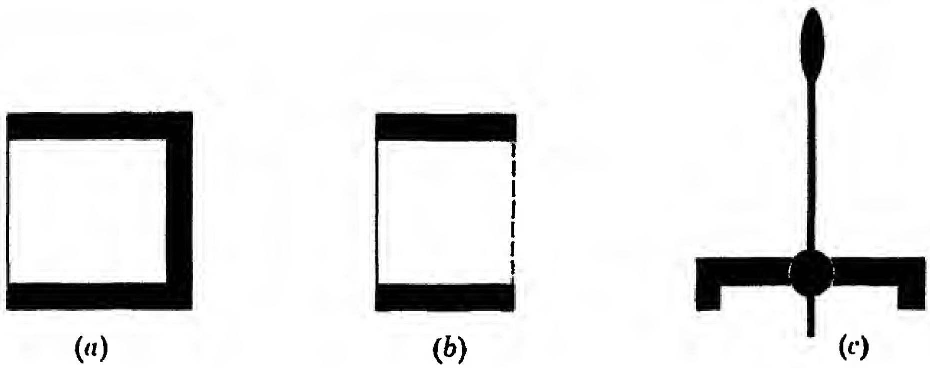



Fig. 1. Diagrams illustrating in section the essential structure of (*a*) a pressure receiver, (*b*) and (*c*) displacement receivers. In (*a*) and (*b*) the diaphragm (full line) and in (*c*) the hinged vane are supposed to be connected to recording apparatus which will register their displacement with respect to the casing (solid black). The dotted line in (*b*) indicates that the back of the casing is transparent to sound.

It is obvious from the foregoing account that such propagated disturbances can be detected by an instrument of appropriate sensitivity and speed which is responsive *either* to pressure changes *or* to displacements of the medium. An instrument of the former type is comparable in principle to an aneroid barometer; one of the latter type is comparable in principle to a wind-gauge.

The essential structure of an instrument for detecting pressure variations is shown in Fig. 1*a*. It consists of a massive chamber closed by a stiff diaphragm whose excursions can be measured by some recording system (not shown). The conditions of its working purely as a pressure receiver are that the walls of the chamber shall be

[1] A further correction is necessary if the displacement is so rapid that the pressure changes are no longer sensibly adiabatic (Herzfeld, W. F. & Rice, F. O. (1928) *Phys. Rev.* **31**, 691).

opaque to sound, that the diaphragm shall be so stiff that its displacement is vanishingly small and that neither the diaphragm nor the chamber shall be resonant to the sound frequencies under consideration. If these conditions could be fulfilled, which is only approximately possible in practice, the excursions of the diaphragm would be proportional to the pressure amplitude and therefore to the square root of the intensity irrespective of frequency. Moreover, since pressure is a scalar quantity, the orientation of the instrument with respect to the direction of propagation of the sound is a matter of indifference, provided that the pressure is uniform over the surface of the diaphragm, i.e. the instrument is small compared with the wave length of the sound. Most of the microphones in common use *and probably the middle ear and tympanic membrane of a mammal* resemble such a pressure receiver in their behaviour.

The essential structure of a displacement receiver is shown in Fig. 1 *b*, *c*. The diaphragm or moving vane should ideally be without mass, and the hinge or suspension completely pliant so that the moving parts follow the movements of the air exactly. If these conditions could be fulfilled, the excursion of the diaphragm or vane would be equal to the normal component of the displacement amplitude and so proportional to $\sqrt{I}$ for constant frequency and to $1/\omega$ for constant $I$. Displacement being a vector quantity, the maximum effect is produced by sound whose incidence is normal to the plane of the diaphragm or vane. The ribbon microphone is a displacement receiver of this kind. And, as will be shown, *it is probable that all insect auditory organs are displacement rather than pressure receptors.*

## II. THE TYMPANIC ORGANS OF INSECTS

Tympanic organs are paired structures which occur in the Orthoptera, Hemiptera and Lepidoptera. They consist always of a thinned region of the exo-skeleton, the primary tympanic membrane, associated with air sacs and a chordotonal organ.

In the Lepidoptera they are known to occur only in the superfamilies Noctuoidea, Geometroidea and some of the Pyraloidea. They are found only in the imagines. They may be in the forepart of the abdomen (Geometroidea, Pyraloidea) or in the metathorax (Noctuoidea).

In the Hemiptera they are only found in the Cicadidae.[1] They occur in the abdomen, and are probably only functional in the adult.

In the Orthoptera they occur in most species of Acridiidae, Tettigoniidae and Gryllidae. In the first of these families they occur in the abdomen, in the second and third at the proximal end of the tibiae of the first pair of legs. Here also, though they may be visible in late nymphal instars, they are only fully developed and probably only functional in the adult.

The primary tympanic membrane, when it is situated on the body, generally lies in a cleft which forms an external meatus and may be further protected by the folded wings or the femur of the third leg or both. Associated with the tibial tympanic

[1] The so-called tympanic organs of *Corixa*, *Plea*, etc., are omitted from this discussion owing to the scantiness of evidence as to their function, but see Graber (1882 *b*), Hagemann (1910) and Wefelscheid (1912).

organ there were generally two tympanic membranes on opposite aspects of the tibia. In many genera these membranes are protected by heavy folds of cuticle which enclose them save for a small pore or slit (e.g. *Decticus*, Figs. 2*a*, 4*e*). In *Gryllus*, where both membranes are completely exposed, but the posterior one is both larger and more delicate, there is a protective device which seems never to have been described. There is on each tibia a hair sensillum with a very long and slender, though stiff, hair directed towards the rear and situated immediately below the membrane (Fig. 2*b*), and in the intact animal, a slight touch on this hair evokes an immediate avoiding response. This hair sensillum resembles certain others which are present in all instars, but it is developed in this position only when the posterior tympanic membrane develops, i.e. in the last larval instar and the adult.

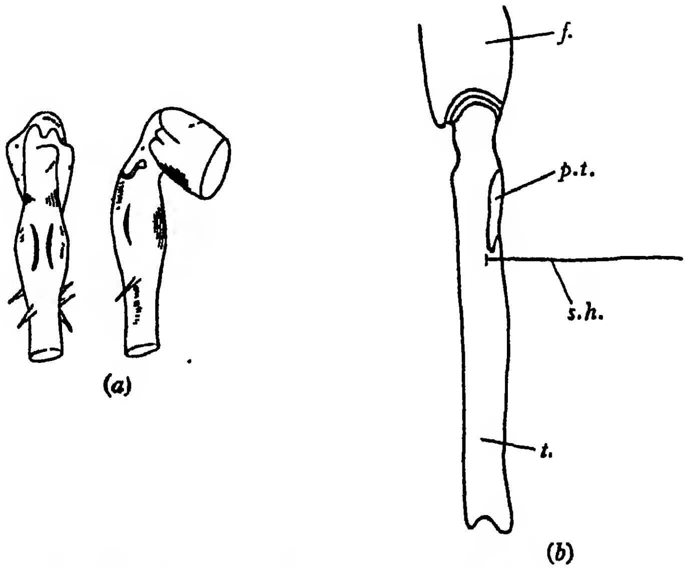
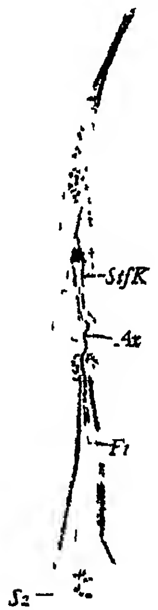



Fig. 2. (*a*) Side and rear views of the tibia of *Decticus* (Tettigoniidae) showing the slit-like openings of the tympanic cavities (after Schwabe, 1906). (*b*) Outline drawing of the tibia of *Gryllus* seen from the side and slightly behind: *p.t.* posterior tympanic membrane; *s.h.* long hair of the hair sensillum; *f.* femur; *t.* tibia.

The chordotonal organs may be directly apposed to the inner aspect of the primary tympanic membrane (Acridiidae, Lepidoptera, Fig. 4*a*, *c*), or applied to its edge (cicadas, Fig. 4*d*) or not directly in contact with it (Tettigoniidae, Fig. 4*e*, Gryllidae). They may contain large numbers of sense cells (1500 or more in cicadas) or few (2–4 in moths). The fundamental structure of the sensory elements of a chordotonal organ is similar in all. Each sensillum consists of three cells, the primary sense cell and two other cells (Fig. 3). The primary sense cell is characterized by inclusions, the axial rod and scolopale, which are constantly present though the proportions are variable in different species. It would appear probable that excitation of the primary sense cell is occasioned by displacement of the axial rod with respect to the scolopale.

As was pointed out in the Introduction, chordotonal sensilla occur throughout the insects, in larvae as well as adults, though tympanic organs occur only in adults and are sporadically distributed. It seems relatively certain, although there is no direct evidence, that the primitive function of chordotonal sensilla is a proprioceptive one—to register the displacement of one part of the skeleton with respect to another (cf. Hertweck, 1931). The development of a special proprioceptive function as in the wing bases and halteres of Diptera or an exteroceptive function as in tympanic organs and perhaps in Johnston's organ is a subsequent adaptation of a




(a)

(b)

Fig. 3. (a) Chordotonal sensillum from tympanic organ of *Mecosthetus* (type with attachment to exo-skeleton only at the distal end): *Sz*, sense cell; *Ax*, axial fibre; *StfK*, scolopale; *Fi*, neurofibril of sense cell. (b) Chordotonal sensillum from tympanic organ of *Cicadetta* (type with two points of attachment to the exo-skeleton). ((a) From Schwabe, (b) from Vogel, 1923.)

pre-existing system to meet a special need. The presence of elaborate chordotonal organs in the tibiae of Hymenoptera which have no tympanic organs and in the tibiae of the second and third legs of tettigoniids and gryllids in a position corresponding to that of the tympanic organs of the first pair is very suggestive. It may well be the case that the tibial chordotonal organs were developed as receptors for vibratory stimuli transmitted through the substrate, before association with a tympanic membrane rendered them more specifically sensitive to air-borne sounds (cf. Autrum, 1936, for a review of the evidence for the sensitivity of ants to vibrations of the substrate).

The presence of air sacs, large expansions of the tracheal vessels in association with the tympanic membrane, is characteristic of all tympanic organs. Their primary purpose is, no doubt, to decrease both the damping and the effective inertia of the membrane by providing it with a backing of air instead of tissue fluids. The air sacs and their associated spiracle which is generally enlarged have often been compared functionally with the cavity of the middle ear and the Eustachian tube of mammals. It is particularly noticeable, however, that there is either a secondary tympanic membrane (Lepidoptera, Fig. 4*c*, tettigoniids, Fig. 4*e*, gryllids), or else the air sacs are so arranged that a series of air chambers connects the two primary tympanic membranes directly (cicadas, Fig. 4*d*, acridiids, Fig. 4*b*). Sound therefore in all cases may reach the primary membrane from either side. This type of architecture is in sharp contrast with the mammalian arrangement where the tympanic membrane covers an opening of a chamber, the middle ear, whose walls are everywhere thick and practically opaque to sound; and it can hardly be doubted that it is of functional importance since it is attained in all the tympanic organs of insects though by different means in different cases. Such an arrangement suggests for one thing that the tympanic membrane responds rather to displacement of the air than to the pressure change, a belief which is supported by experimental evidence (see pp. 122–123).

As was intimated in the Introduction, the first attempt at a thorough analysis of the function of tympanic organs was that of Regen. His experiments were limited to the cricket, *Lyogryllus campestris*, and a long-horn grasshopper, *Thamnotrizon apterus* (= *Pholidoptera aptera*), in both of which the tympanic organs are situated in the tibiae of the forelegs. To him belongs the credit of introducing electrical sound reproduction into the study of insect hearing and in 1913 he showed that the female cricket was attracted to a telephone which transmitted the chirping of a male in another room. This response (1923) was obtainable only from the adult but *unmated* female, though both sexes are provided with tympanic organs in the adult and in the last larval instar. The dependence of the response not only on the sex but on the physiological condition of the experimental animal probably may be taken as explaining a great deal of the conflicting evidence in older work.

His approach to *Thamnotrizon* was different (1914). He had observed that two males in the same vicinity showed a tendency to sing in concert. The analysis of such a concert showed considerable complexity. There was a *Vorspiel* and a *Nachspiel*, but almost always an intermediate period in which the chirps of the two performers alternated in a regular manner. The very occurrence of such concerts demonstrated that hearing occurred, and Regen took advantage of it (1926) to see whether a male could be persuaded to alternate with an artificial partner. At first he was completely unsuccessful and the result of his intervention was the immediate suppression of the song. But he found that by taking males which had only just entered on the adult condition, he could persuade them to alternate with a wide variety of instrumental noises. Important consequences of this fundamental observation will be considered below. Here it is only necessary to say that it indicated a range of sensitivity from somewhere in the neighbourhood of 400 c./sec. to more than 28,000 c./sec. No determinations were made of the liminal intensity at any frequency. In both the

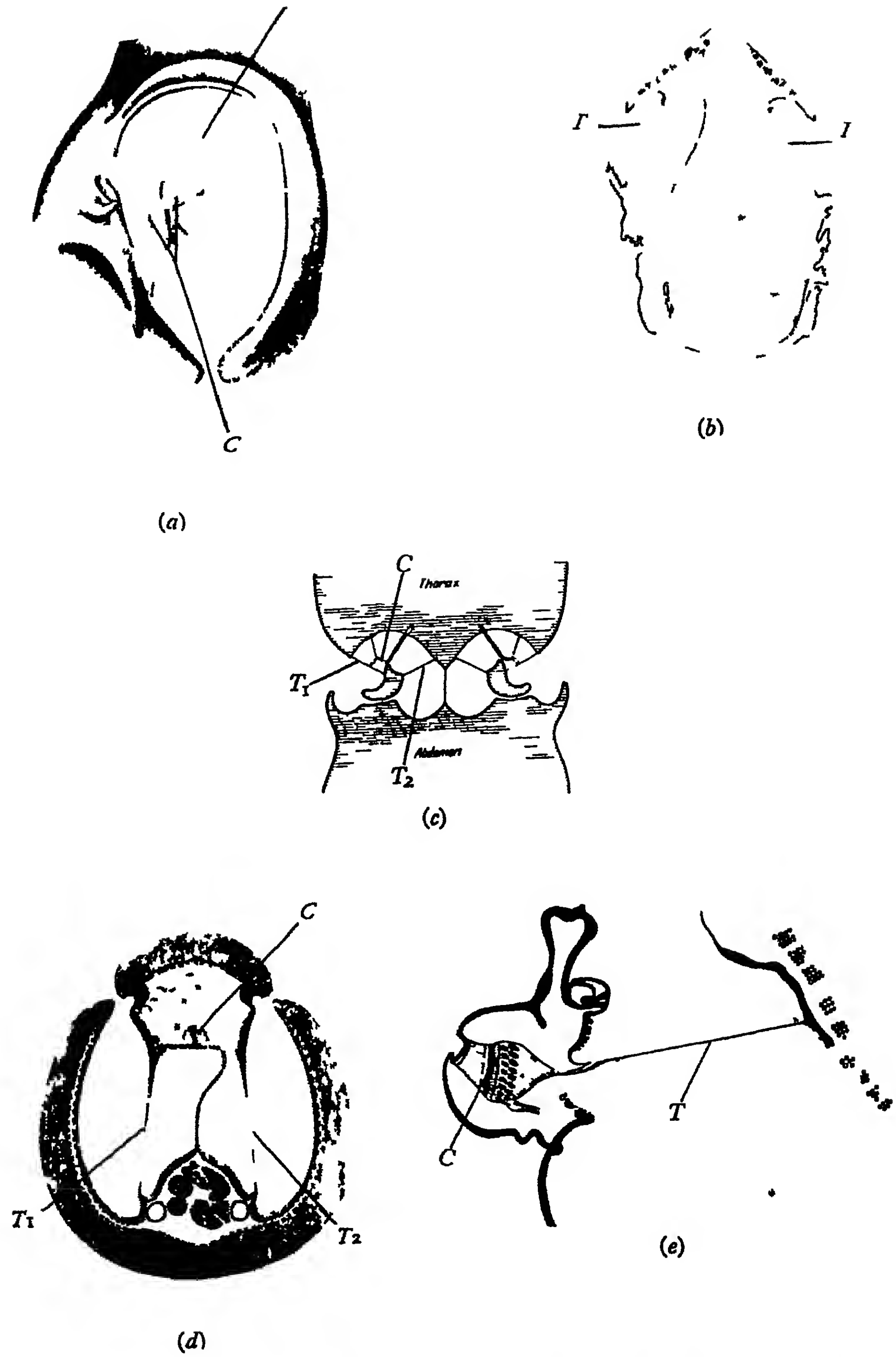



Fig. 4 (*a*) Inner aspect of the tympanic organ of *Mecosthetus* (Acrididae) (*b*) Transverse section through the abdomen of *Mecosthetus* at the level of the tympanic organs, showing the chain of air sacs connecting the tympana (*c*) Schematic horizontal section through a noctuid moth, showing the arrangement of primary and secondary tympana and the connecting air sacs. (*d*) Horizontal section of tympanic organ of *Cicadetta*. The internal air space is continuous with that behind the opposite tympanic organ. (*e*) Transverse section of tympanic organ of *Decticus*. *T*, tympanic membrane; $T_1$ and $T_2$, primary and secondary tympanic membranes, *C*, chordotonal organ. ((*a*), (*b*) and (*e*) after Schwabe, (*c*) after Eggers and (*d*) after Vogel.)

cricket and the grasshopper (1914) he proved that bilateral extirpation of the tympanic organs abolished the normal responses, though some residual sensitivity to sound could be demonstrated.

Wever & Bray (1933) inserted electrodes into the femur of the foreleg of crickets and tettigoniids. The electrodes were connected to an amplifier and speaker so that the electrical response in the nerve of the leg was made audible when the tympanic organ was excited by tones from an oscillator and other sound sources They found that the adequate range of frequencies for the cricket was 250–10,000 c./sec and for a tettigoniid (unnamed, but either *Amblycorypha oblongifolia* or *Pterophylla camellifolia*), 800–45,000 c./sec. They noted, moreover, that the response to all adequate frequencies was asynchronous, i.e. the nerve impulses were apparently random, and the character of the discharge was not affected by the frequency of the incident stimulus.

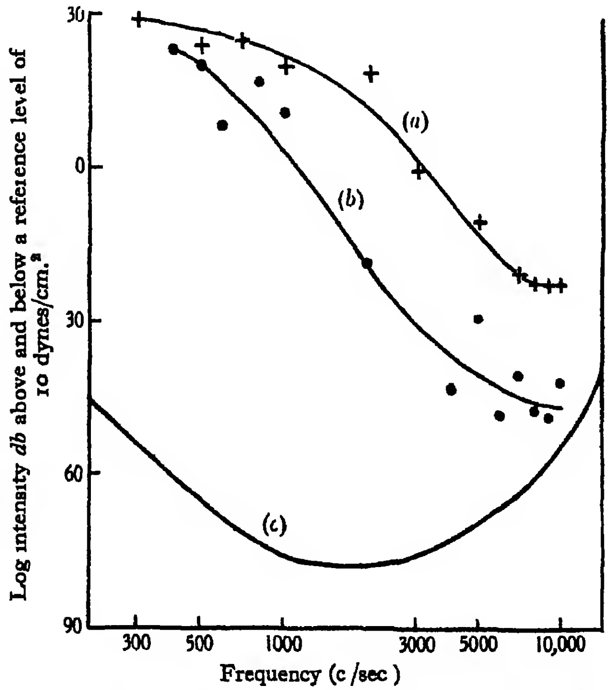



Fig 5. (*a*) Threshold curve for tympanic organs of *Arphia sulphurea* averaged from Wever's figures for four individuals (*b*) Threshold curve for a single isolated tympanic organ of *Locusta* (*c*) For comparison mean human threshold subjectively determined, from Wegel (1932) In this figure and subsequently the threshold of an auditory organ determined oscillographically is that intensity of sound which produces in the auditory nerve a just perceptibly greater discharge of impulses than is evident when no sound impinges on the organ

Pumphrey & Rawdon-Smith (1936*b*), in an examination of the response of the abdominal tympanic organ of the locust (*Locusta migratoria migratorioides*) by a similar method, determined the threshold intensities for frequencies up to 10,000 c./sec. Their data and those of Wever on another acridiid, *Arphia sulphurea*, converted to similar ordinates are shown in Fig. 5. Wever placed his recording electrodes "on or

near" the metathoracic ganglion. Pumphrey & Rawdon-Smith dissected out the tympanal nerve and freed it from its central connexions, thereby avoiding reflex activation of the tympanal muscle and other disturbances of central origin. It is very likely that the consistently higher threshold obtained by Wever was due to the difference of technique rather than to any marked dissimilarity in the properties of the tympanic organs in these two genera. The function of tympanal muscles (found not only in acridiids but in Lepidoptera and Hemiptera) is not known, but it is probably similar to that of the tensor tympani of mammals. The nervous response was asynchronous (1936*c* and Wever, 1935), like that of the tympanic organ of the cricket.

In sharp contrast with the mammalian ear and with some other auditory mechanisms in insects, the tympanic organ of the locust does not seem to be susceptible to fatigue.[1] No diminution in the response to a 2000 c./sec. tone could be detected after half a minute's continuous stimulation (1936*c*).

The available information about the tympanic organs of the Lepidoptera is very slender. The fact of hearing seems to be established. A number of noctuids and geometrids have been shown to react to artificial sounds, Stobbe (1911), Turner (1914), Turner & Schwarz (1914), Eggers (1925, 1926).

According to Eggers, who used as a stimulus a high pitched squeak produced by twisting a glass stopper in a bottle, a reaction was obtainable exclusively from Lepidoptera which possessed tympanic organs. Turner & Schwarz record reproducible responses of *Catocala* spp. to high-pitched Galton whistles, but in one species, *Catocala ilia*, responses were obtained to an organ pipe (256 c./sec.) and Turner describes similar responses in several saturniids to pipe notes of the same frequency. *Saturnia* has no tympanic organs and in *Catocala* it is unlikely that the response to notes of relatively low pitch is mediated by tympanic organs, in view of what is known of the frequency range of such organs in other insects. Probably the receptors involved are sensory hairs (see below). On the other hand, responsiveness to tones of high pitch and to "squeaks" seems to be a prerogative of moths possessing tympanic organs.

Here, as in Orthoptera, functional tympanic organs are probably connected with a mating response. According to Eggers they are absent in the flightless females of some noctuoid genera. Peter (1912) describes a case (*endrosa* var. *ramosa*) where the male makes a crackling noise in flight to which the female responds by shaking her wings and body. Quite possibly the males in other genera make noises inaudible to human ears which may either guide the female or by exciting her to movement make her conspicuous to the male who is seeking satisfaction. Some authors (e.g. Turner) however, have believed that the flight reaction is without sexual significance. They have noted that the sounds which are most effective in producing the reaction resemble in pitch the squeaks of bats and field mice and have concluded that auditory

[1] This refers to the response to pure tones. The "synchronous" response to an amplitude modulated "carrier" (p. 121) shows "alternation" and "equilibration" exactly analogous to the phenomena observed in the response of the cercus (p. 125) and the mammalian cochlear nerve in response to pure tones.

sensitivity in this range may enable the insect to escape these animals which are its natural enemies. Turner & Schwarz (1914) and Eggers (1928) give good summaries of the earlier literature.

Information about the auditory powers of the cicadas is even more scanty. Fritz Müller has described an alternating concert in South American cicadas and Leydig observed that the cicadas in the vineyards round Würzburg were excited to more vigorous singing by the drums of the garrison on parade (see Eggers, 1928, p. 327). Fabre, however, failed to produce any effect on the cicada song in Provence by the discharge of the largest cannon which he was able to borrow (1911).

## III. THE QUESTION OF FREQUENCY DISCRIMINATION

So long as the attainments of the human ear are taken as a yardstick and the competence of hearing organs in lower animals is judged by the degree to which they approach the human ear structurally and functionally, it is possible to question whether insects are able to hear. Even if some dim sense of hearing is allowed to them it is arguable, *a priori*, (and it has been so argued) that any discrimination of the quality of sound is impossible in animals which possess nothing remotely resembling a cochlea. But such an argument flies in the face of the facts. It can hardly be questioned that Regen's experiments demonstrate the ability of *Thamnotrizon* to discriminate between sounds which differ qualitatively irrespective of their intensity. To recapitulate, Regen found that males which had just entered on adult life could be induced to sing in rhythmic concert with artificially generated sounds over an enormous frequency range. Those adults, however, which had already learnt to sing in concert with their brethren immediately detected any attempt at imposture and were reduced to silence no matter how closely (by human criteria) the artificial sounds approximated to the natural song. It could conceivably be contested that some defect of rhythm in the artificial song rather than a departure from the frequency characteristic of the natural chirp was responsible for its detection; but this argument is conclusively answered by Regen's observation that when the artificial partner intervened the cessation of song was immediate. No such immediate cessation was observed when, as sometimes happened, one partner got out of step in the natural alternating concert. It is, therefore, certain that this grasshopper possesses a high degree of discrimination of qualitative differences in sound.

The observations on the cricket are in apparent conflict with the above, for Regen states that the unmated female recognizes and orientates herself towards the telephonically transmitted chirp of the male even though the latter's song was so distorted by transmission as to be quite unrecognizable to the human ear.

If it is to be assumed that the mechanism of recognition is similar in the cricket and the long-horn grasshopper (and there is no reason to doubt it), it becomes apparent that the qualities of sound which facilitate recognition in these animals are not the same as those which facilitate recognition by the human ear.

It is worth while therefore to attempt to banish all preconceptions, not only those founded on human sensory experience but also those based on our knowledge

of human and mammalian physiology, and to consider what solutions are possible within the restrictions imposed by the physical nature of insects, in particular their magnitude, and by what is known of the physiology of *their* receptive organs.

It is extremely probable that the principal function of tympanic organs is to enable detection and recognition of the stridulation (or other noises) produced by other individuals of the same species. It is a matter of common observation that such noises are of high pitch close to the upper limit of the human auditory spectrum; and, because of their small size, it would in fact be impossible for insects to radiate sounds of low frequency with efficiency. Moreover, as has been shown, tympanic organs do not respond to sounds of low frequency and are most sensitive in the region of 5000–20,000 c./sec. It can be accepted, therefore, that such faculties of discrimination as the insect may possess must be exercised upon sounds whose component frequencies are of this order.

There seem to be only two possible methods by which sounds which differ qualitatively can be discriminated irrespective of their relative intensities. The first is that different nerve fibres shall be excited by the different frequency components of the sound. This is the method of harmonic analysis which is believed with good reason to be the method by which frequency discrimination occurs in the mammalian and avian ear. The only alternative possibility is that the same nerve fibres shall be excited in a different way by different sounds. Since the only variable function of the nerve fibres is the *frequency* with which they transmit impulses, this alternative possibility can be stated in the form that different sound qualities must be represented in the individual fibres of the auditory nerve by different temporal patterns of nervous impulses.

The first alternative is ruled out by the structure of the tympanic organs. It is true that the graduation in size of the linearly arranged chordotonal sensilla in the tympanic organs of tettigoniids and gryllids has at times prompted fanciful comparisons with the fibres of the basilar membrane of the cochlea. But it is not credible that the sensory end-organs shall themselves be resonant elements resembling stretched strings, for this would imply that while they are already under considerable tension (that of the basilar membrane fibres is believed to be several tons per square inch in the basal turn of the cochlea) they would yet be sensitive to the minute increments of tension resulting from very small displacements of the tympanic membrane. In the cicadas the chordotonal sensilla are in a single mass and are approximately of equal size. In the acridiids it is true that the sensilla are arranged in three groups attached to different points on the tympanic membrane. Since the latter is not homogeneous and the thickness and stiffness vary considerably from side to side, it is conceivable that frequency discrimination might occur in a manner analogous to that put forward for colour discrimination in photopic vision, viz. that the displacement of different parts of the tympanic membrane might differ relatively for different frequencies, and consequently the relative degree of excitation of the three groups of sensilla might vary with frequency. Pumphrey & Rawdon-Smith (unpublished) succeeded in destroying one of these groups and could find no significant difference in the threshold curves determined before and after the opera-

tion. This observation can hardly be taken as proof that frequency discrimination does not occur, for, on the analogy with Hecht's theory of colour vision, the threshold curves of the three groups of sensilla might differ by an amount too small to be detected by so crude an approach. The balance of probability is however very much against frequency discrimination in insects by the method of harmonic analysis.

The second alternative must, therefore, be closely scrutinized, namely that different sound qualities are represented by different frequencies of impulses in the auditory nerve. It is clear from the evidence quoted above that no detectable pattern of impulses is produced by pure tones. It is conceivable though very unlikely that regularities might exist but be masked by appearing independently in different fibres. No direct disproof is possible until records have been obtained from single fibres. However, if this possibility is neglected, it becomes evident that insects cannot discriminate between *pure tones* in the band of frequencies to which they are sensitive except on the basis of differences in intensity. It is necessary, therefore, to look for some other basis for the discrimination of the quality of sound, and a hint is given by the nature of the sound-producing mechanisms of insects. In the Orthoptera sounds are produced by rubbing a toothed ridge to and fro across the edge of a wing or skeletal element which acts as the radiating surface. In the cicadas the characteristic song is produced by the vibrations of a diaphragm plucked by a special group of muscles. The detailed analysis of the wave form of such noises has not yet been carried out, but it is fairly evident that they must consist of a high frequency corresponding to the resonant frequency of the wing or diaphragm or other radiating surface and that this high frequency must undergo modulation[1] in amplitude at a lower frequency corresponding to the rate of incidence of successive teeth of the scraper and to the change of direction of the latter, or, in the case of the cicadas, to the frequency of the muscular contractions which excite the diaphragm. In terms of an electric analogy such a system is equivalent to a damped resonant circuit excited periodically by pulses from a relaxation oscillator.

That modulation of the resonant frequency is occurring in the production of sound by insects is often evident to the human ear from the trill-like character of the sound. That amplitude modulation[1] of a high frequency note to which the tympanic organ of an insect is sensitive may produce a characteristic temporal pattern of impulses in its auditory nerve has been demonstrated by Pumphrey & Rawdon-Smith (1939). It is reasonable, therefore, to conclude that it is the modulation pattern which characterizes the sound for an insect hearer, since the corresponding nervous pattern of impulses depends only on the frequency (and perhaps the form) of the modulation and is independent of the modulated frequency provided that the latter is within the audible range. A human observer, by contrast, is very sensitive to changes of the modulated frequency and quite insensitive over a wide range to changes of the modulation frequency. It is, therefore, easy to understand how

[1] When a wave train of substantially constant frequency experiences a change in amplitude it is said to undergo *amplitude modulation*. This term is generally used of periodic amplitude changes, and the frequency of the change in amplitude is then known as the *modulation frequency*.

insects may recognize as similar sounds which are quite different to human ears and conversely may distinguish sounds which to human ears are identical.

It is important to realize that, if the theory outlined above is correct, what is occurring in the tympanic organ is not "frequency discrimination" as ordinarily understood. It is true that a high sinusoidal frequency ($n$) modulated sinusoidally in amplitude at a lower frequency ($m$) can be resolved into three unmodulated component frequencies of $n$, $(n+m)$ and $(n-m)$. Such analysis is of extreme importance in *human* acoustic theory for the very reason that the cochlea is a harmonic analyser and that the locus of excitation on the basilar membrane depends not primarily on the wave form but on the frequency components of the sound irrespective of their phase relationships. But the possibility of harmonic analysis of sound of any wave form has no special relevance in considering auditory organs which differ from the human ear in possessing no peripheral mechanism which is differentially resonant and which are free, therefore, from the advantages and defects of such a mechanism. A harmonic analyser could not respond to the modulation frequency as such because there is no real component of such a frequency in the transmitted sound wave, just as there is no component of audio-frequency in the electro-magnetic wave train produced by a wireless telephone transmitter. That volleys of impulses appear in the insect's auditory nerve at the modulation frequency demonstrates that the tympanic organ is acting rather as a rectifier like the "detector" of a wireless set. Such rectification is, of course, not peculiar to the tympanic organ. It can be shown to occur in any instrument employing an asymmetrically loaded diaphragm, including the human ear. The difference is that in the human ear it is a consequence of a mechanical imperfection, which has the result of producing various auditory illusions, such as "summation" and "difference" tones, whereas in insect hearing it is probably the prerequisite of the discriminative process.

## IV. THE QUESTION OF LOCALIZATION

Little information on this point is available except from the work of Regen. In 1923 he showed that unmated female crickets oriented towards a chirping male at a distance of 10 m. or more, and that on an unobstructed floor their movement towards the male was substantially along a straight line, though there were continual small divergences to left and right. It would seem extremely unlikely that at such distances the differential intensity of the sound reaching the two tympanic organs would be adequate for orientation; it would not exceed 0·00002% for a divergence of 20° at 10 m. Moreover, females deprived of one tympanic organ reached the male, though by a much more erratic path, and Regen mentioned no tendency to execute circus movements about the male as might be expected if either differential intensity or phase were the operative agents on the intact animal. It is, therefore, indicated that each tympanic organ is itself a directional instrument, i.e. it responds to displacement or velocity which are vectorial functions of sound rather than to pressure. This is in conformity with its structure (p. 115). Moreover, Pumphrey & Rawdon-Smith (unpublished), using a monophasic transient as a test

stimulus, have determined the threshold amplitude required to stimulate the isolated tympanic organ of the locust as a function of the angle of incidence. In Fig. 6 the sensitivity (reciprocal of the logarithm of threshold amplitude) has been plotted against direction of incidence, so that the distance of a point from the origin represents the sensitivity for sounds propagated along the line joining the point to the origin.

It is clear, therefore, that this tympanic organ has directional characteristics. This directional sensitivity is noteworthy because the assumption has so often been

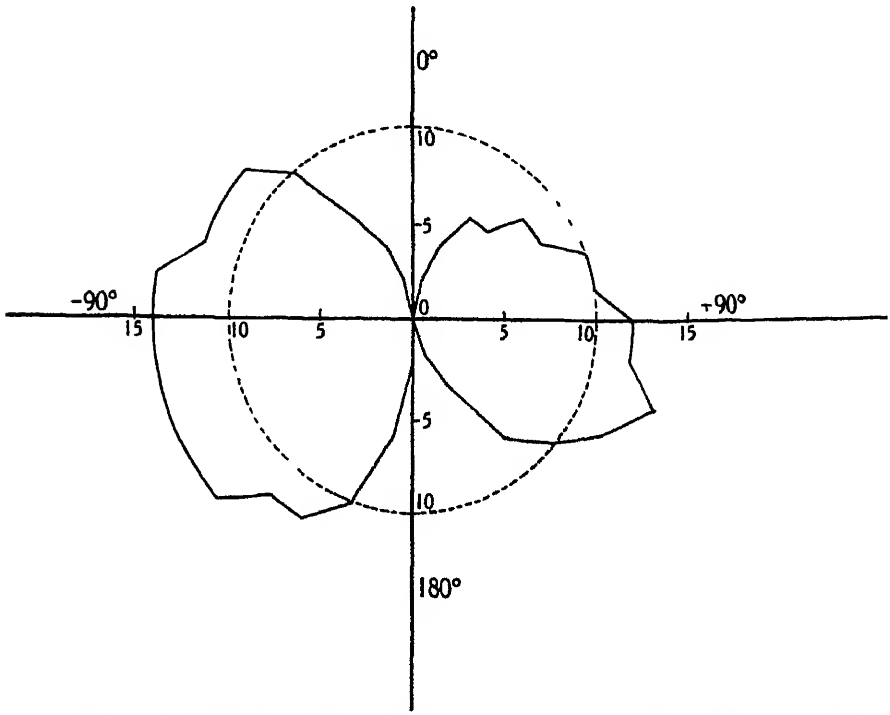



**Fig. 6. Sensitivity of an isolated tympanic organ of *Locusta* plotted on polar co-ordinates as a function of the direction of incidence of the test stimulus. Sensitivity (log reciprocal of threshold amplitude) is plotted radially and the minimum sensitivity is arbitrarily taken to be zero. The line 0–180° lies in the sagittal plane of the animal, and for angles of positive or negative sign the test stimulus is incident on the external or internal aspect of the tympanic organ respectively.**

made in the past that tympanic organs, like mammalian ears, are pressure receptors. For example, Autrum (1936), in developing a theory that auditory organs in arthropods are velocity (*Schallschnelle*) receptors, says: "Die entwickelte Theorie gilt für allen Arthropoden, *soweit sie keine Trommelfelle besitzen.*"

It is also noteworthy that since monophasic transients were used and the curves in Fig. 6 are nearly symmetrical about the sagittal plane, the tympanic organ is itself nearly symmetrical and responds both to a "push" and a "pull". This was independently confirmed by reversing the direction of the transient for a fixed direction of incidence; no difference either in the form of the nerve discharge or in

the latency could be detected when a negative pulse was substituted for a positive. This behaviour is also in sharp contrast with the behaviour of vertebrate auditory organs.

## V. HAIR SENSILLA AS AUDITORY ORGANS

As was stated above the responses of insects to sound are by no means limited to insects possessing tympanic organs.

Minnich (1925, 1936) has investigated the response of a considerable number of lepidopterous larvae. In the earlier work he used the hairy caterpillars of *Vanessa antiopa*. These respond to adequate acoustic stimulation by a contraction of the dorsal longitudinal muscles which in the intact animal raises the anterior third of the animal from the substrate. The response can, however, be readily detected in fragments of the caterpillar and Minnich used this fact to show that the receptors were generally distributed over the body, though they were less dense, or less susceptible, in the posterior third. Minnich used as his ordinary stimulus the decremental note produced by a tuning-fork whose arms were pressed together a standard distance and suddenly released. The upper limit of frequency to which a response could be obtained was in the neighbourhood of 1000 c./sec., though it could be pushed somewhat higher by using higher intensities. The lower limit was not reached at 32 c./sec. The response was greatly reduced or abolished when the hairs covering the caterpillars were clogged with a water-spray or with flour and returned when the water evaporated or the flour was removed. The response to sound was masked or inhibited by a constant air stream impinging on the animals. Taken together, these last two observations show conclusively that the hair sensilla are in fact the receptors concerned. Minnich believed that the hairs might be resonant structures, and endeavoured to demonstrate this by fatiguing the response to one fixed frequency and then testing for a response to another frequency. If the hairs were resonant, then, since they were different lengths and sizes, it would be expected that different hairs would be excited by the tones, C (256 c./sec.), G (348 c./sec.) and C′ (512 c./sec.), and therefore that fatigue of one group (assuming that fatigue was peripheral and not central) should leave the response of the others unaffected. What he actually found was that fatiguing at a low frequency inhibited the response to higher frequencies, but that fatiguing to a higher frequency left the response to lower frequencies relatively unaffected. This is inconsistent with the resonance interpretation, but is easily explained in the light of the observations of Pumphrey & Rawdon-Smith (below). In 1935 Minnich repeated some of these experiments with a number of other larvae. He found that from all of these a response, sometimes of contraction of the longitudinal dorsal muscles, sometimes of "freezing", could be obtained. It is noteworthy that some of these larvae were relatively hairless, and, though the intensity factor was not controlled, it seems implicit in the text that the threshold for these forms was higher and the range of frequencies to which a response could be obtained about the same. He was unable to confirm Abbott's (1927) observation that the larvae of *Datana perspicua* responded only to certain definite frequencies.

The tentative conclusion to be drawn from Minnich's observations is that the sensitivity to sound is mediated by hair sensilla, certainly in the hairy forms and probably in the relatively hairless forms, that such hair sensilla are not resonant and that they respond more readily to low frequencies than to high.

Pumphrey & Rawdon-Smith (1936*a*, *b*) recorded oscillographically the response of the cercal nerve of a number of Orthoptera when the anal cercus was excited by pure continuous tones, derived from an oscillator and speaker. The function of the cercus was not previously known, though the structure of the sensilla had been described by Sihler (1924), who also by a number of experiments had shown that it was not an olfactory organ as had been surmised by McIndoo owing to the presence of numerous campaniform sensilla. Its responsiveness to sound was discovered accidentally and the remarkable similarity of the oscillograms from the cercal nerve

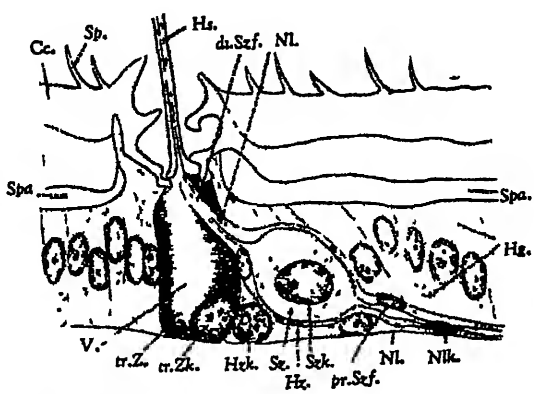



Fig. 7. Section of the base of a long hair sensillum from the anal cercus of *Gryllus* (× 1100). On the same scale the hair (*Hs*) would be from 2 to 3 m. in length. *Sz*, Sense cell with its dendrite (*di. Szf.*) in contact with the hair. (From Sihler, 1924.)

in response to pure tones to those obtained from the cochlear nerve of mammals excited immediate interest. Pumphrey & Rawdon-Smith found that the response was inhibited by entangling the hairs with dust or vaseline and concluded that the responsive end-organs were the long hair sensilla (Fig. 7). These hairs are so lightly hinged that they will visibly move in response to air movements far too slight to be detectable by the human skin or the hairs on the back of the hand. They can also be excited to visible movement by sounds of adequate intensity and show no special preference for a particular frequency, i.e. they are non-resonant.

The nervous response is synchronous at least initially, up to a frequency of 800 c./sec. (Fig. 8) and like that of the mammalian cochlear nerve may show "alternation" (Davis, 1935). The threshold curve is of considerable theoretical interest (Fig. 9) as it shows that these hair sensilla act as pure displacement receptors over the range Pumphrey & Rawdon-Smith were able to investigate. In Fig. 9 the heavy line which fits the experimental points very well corresponds to a constant displacement amplitude of 560 A. Assuming that the hair acts as a rigid lever, it is possible

to form an approximate estimate of the threshold displacement of the dendrite at the base, and to conclude that it cannot greatly exceed 0·5 A., and may be much less. It would seem likely, therefore, that the limit of useful sensitivity has been closely approached. Indeed, it is possible that the residual activity always detectable in the

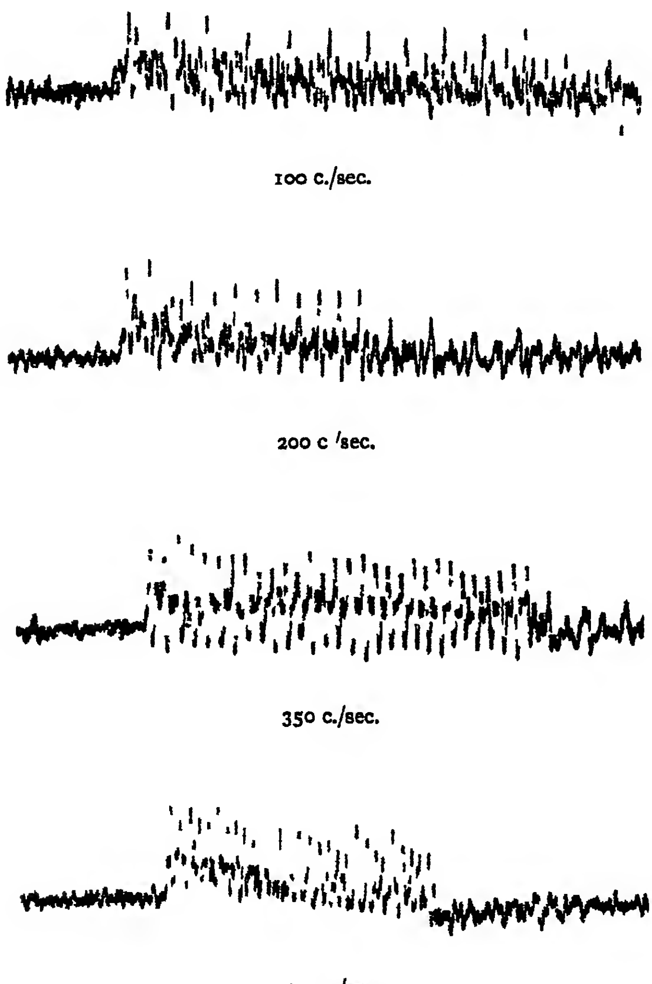



Fig. 8. Oscillograms of the response of the cercal nerve of the cricket, when pure tones of various frequencies are incident on the cercus. At these frequencies the response is synchronous.

cercal nerve in surroundings made as silent as possible is due to occasional excitation of the end-organs by Brownian agitation of the molecules in their vicinity.

These figures are for the cricket, but very similar responses are obtainable from the anal cerci of the cockroach (Pumphrey & Rawdon-Smith, 1936 *a*) and the locust.

If it is legitimate to assume that the hairs on Minnich's caterpillars resemble

those of the cercus in their properties, a ready explanation is available for his experiments on fatigue. For stimuli of equal intensity, those of lower frequency will be much louder to the experimental animal than those of higher frequency and may consequently be presumed to have a more fatiguing effect. It is not, therefore, necessary in this case to postulate resonant elements.

One significant difference may be noted between these hair receptors and tympanic organs. The latter are characteristic of the adult condition, and it is probably fair to say that their development is always connected with a mating response. The

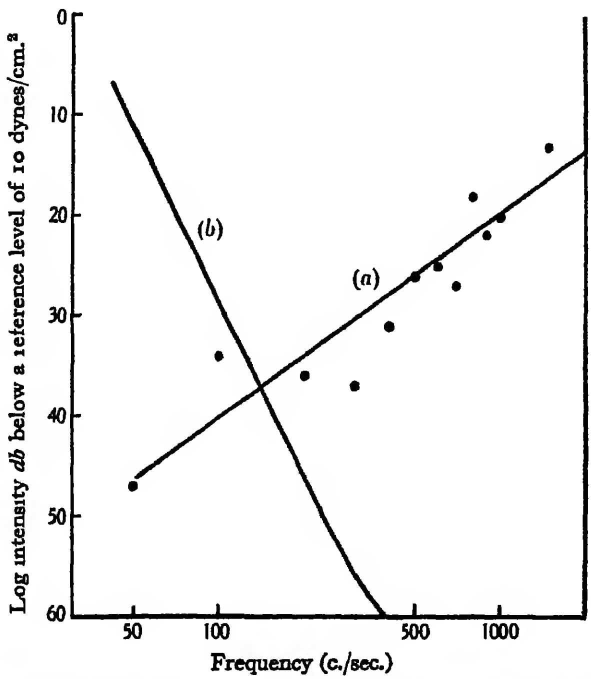



Fig. 9. The points represent experimentally determined thresholds for a cercus preparation of *Gryllus* for pure tones at the indicated frequencies. The heavy line (*a*) corresponds to a constant displacement amplitude of 560 A. The curve (*b*) is drawn from Wegel's figures for the human threshold of hearing.

hair sensilla on the other hand are characteristic of all stages of development. In the cricket the hair sensilla of the cercus are present in the early larvae and are certainly functional in the smallest instars which it was possible to dissect.

## VI. OTHER ACOUSTICALLY SENSITIVE RECEPTORS

Pumphrey & Rawdon-Smith (1936*b*) have recorded a response in the abdominal segmental nerves of the locust in response to sounds of frequencies in the neighbourhood of 1400 c./sec. The form of the response curve (Fig. 10) suggested that some fairly sharply tuned resonant structure was involved and they attributed the response to sensory hairs sufficiently short and stiff to be resonant to a frequency of 1400 c./sec. No positive evidence for this hypothesis was obtained, and it is perhaps

more probable that the response was due to segmental chordotonal sensilla.[1] This is suggested by the fact that the number of active fibres in each segmental nerve appeared to be extremely few (probably two). In these preparations it was very evident that the frequency of nervous impulses bore no relation to the frequency of the incident sound but increased with increasing intensity. These preparations showed little trace of fatigue in response to continuous stimulation, therein resembling the chordotonal sensilla of the tympanic organ and differing from the preparations of auditory hair sensilla.

Autrum (1936) has investigated the auditory sensitivity of ants. His experiments seem to show conclusively that the ants he studied (*Formica rufa*, *Myrmica* spp.,

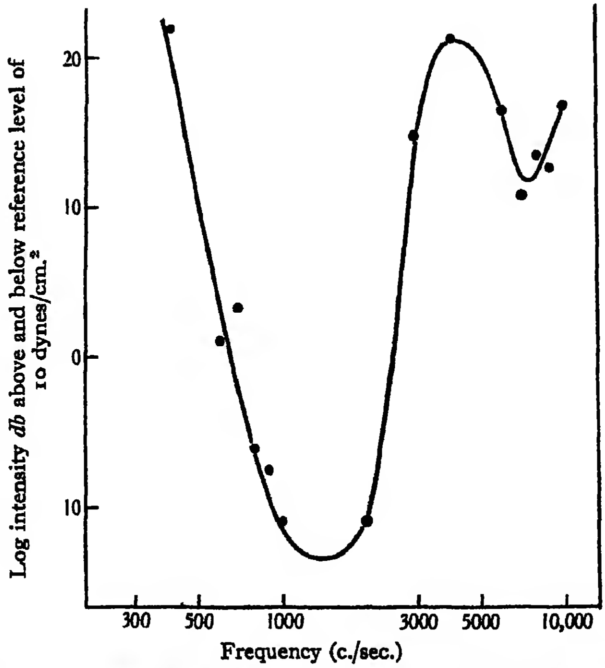



Fig. 10. Threshold curve for acoustically sensitive end-organs with afferent fibres in the segmental abdominal nerves of *Locusta* (? segmental chordotonal sensilla). Reference level as in the earlier figures. Note that the absolute maximum sensitivity is much lower than for the other auditory organs.

*Lasius* spp.) are deaf to their own stridulation, but may respond to very loud artificial sounds. His method of demonstrating that the ants responded to displacement of the air (or some function of it) and not to pressure change was ingenious. Sound was directed vertically downwards upon a reflecting surface, thereby setting up standing waves. Ants walking upon the reflecting surface are in a region of maximum pressure change and minimum displacement and such ants did not respond at all to the sound. Other ants suspended upon gauze above the reflecting surface at an antinode where the displacement was maximal and the pressure amplitude minimal responded vigorously. Autrum is incorrect, however, in supposing that his experiments demon-

[1] Segmental chordotonal sensilla have not been demonstrated in the locust, but there are seventy-six pairs in another acridiid, *Melanoplus* (Slifer, 1936).

strate that it is necessarily the velocity (*Schallschnelle*, i.e. the first differential of the displacement) which is the determining factor. Displacement, velocity and acceleration are all maximal at an antinode and this method does not permit of discrimination between them. The sounds used were very loud indeed, 90–110 phons (= $10^9$–$10^{11}$ times the threshold intensity for the human ear at 1000 cycles). It would, therefore, seem very unlikely that air-borne sounds play any appreciable part in the perceptual field of the ant. Autrum seems to believe that the antennae are the receptors involved in his experiments, though no conclusive evidence is given in support of this view. It would seem equally probable that at such intensities the whole ant would be displaced sufficiently with respect to the gauze on which it walked for the excitement of the chordotonal organs in the legs (cf. Fielde & Parker, 1904).

As has been pointed out above, the name "chordotonal organ" has come to be construed in a structural rather than a functional sense. Nevertheless, the presence of such an organ (Johnston's organ) in the base of the antennae of most insects has been taken to imply an auditory function for these appendages. The experimental evidence for such a view is very meagre. The observations of Mayer quoted in the Introduction do not necessarily imply that Johnston's organ is involved, for the long hairs on the antennae of male gnats are probably directly innervated and may respond to sound in a way similar to other long sensory hairs. From the work of Hollick (communication to the Society for Experimental Biology, 1939) it appears that in cycloraphous Diptera Johnston's organ responds to displacement of the antenna by air currents and plays an important part in flight in mediating the reflex adjustment of the wing-beat to the appropriate form for a given air velocity. According to Eggers (1926*a*), the same organ in *Gyrinus* responds to changes in curvature of the water surface. Johnston's organ is, therefore, obviously rather a labile structure and the possibility of its mediating sound reception in some insects can by no means be ruled out. The subject needs further investigation.

## VII. CONCLUSION

The present time is perhaps a happy one for reviewing the experimental findings on the physiology of audition in insects in relation to their behaviour. The brilliant morphological work of generations culminating in the monographs of Schwabe and Eggers enables the experimentalist to approach the subject with almost as full a knowledge of peripheral structures as has the mammalian physiologist. At the same time the experimental period has been short enough for a reviewer to be able to cover it fairly adequately without either rejecting relevant material or writing at intolerable length. It is to be hoped that this review will be helpful in indicating some of the directions in which further experimental work is urgently necessary. In particular, field observations by naturalists who are informed as to the properties of insect auditory organs might be extremely helpful to the experimentalists.

It will be obvious that much remains to be done. It is a great pity that nothing is known of the central connexions of the fibres from tympanic organs in any insect,

still less of the destination of the second and third order neurones. In the case of the cercus of Orthoptera, Pumphrey & Rawdon-Smith (1937) traced oscillographically second order neurones running the length of the cord to the cerebral ganglia, and Pringle (personal communication) has found that the leg muscles of the cockroach are innervated by "fast" fibres which are apparently excited by collaterals of these neurones. Minnich's experiments make it clear that in caterpillars there are local segmental reflex arcs involved in the response to sound. On the other hand, in those insects with tympanic organs in which appropriate sound stimulation leads to more complex responses, it seems fairly certain that there must be a process of integration requiring a more complex neural organization than can be found in the segmental ganglia. But there is as yet no evidence either for or against the existence of auditory centres in the brain.

Again we are still extremely ignorant of the actual wave form of the noises produced by the insects themselves. Until these have been analysed, any assumptions as to the method of "frequency discrimination" must remain largely hypothetical, for it seems fairly certain that in most cases it is in respect of these particular kinds of noises that discrimination is best.

Yet a third limitation is the extremely narrow field over which physiological experimentation has been carried on. There are many groups of insects in which responses to sound have been reliably reported, but which have been omitted or mentioned only in parenthesis in this review, Coleoptera, Hymenoptera, Ephemeroptera, Isoptera, etc. Of particular interest are those cases where the insects make noises which would seem a suitable means of communication. It passes belief that stridulating organs should have been evolved, as has actually been suggested, as a sump for surplus nervous energy, and until it can be demonstrated that such mechanisms serve some biological function other than communication, it is necessary to regard experiments purporting to show that the sounds produced are not perceived by other individuals of the same species with great reserve.

## VIII. SUMMARY

1. The difficulties attending precise definition of auditory organs are discussed and a sketch of the early work on insect hearing is given.

2. A brief account is given of the nature of sound and of the physical conditions which different types of auditory receptor must fulfil.

3. The tympanic organs of insects, though undoubtedly of independent origin in the different groups of insects in which they occur, are shown to have a common structural plan; and the implications of this fact are discussed.

4. An account is given of the important experimental work of Regen which first conclusively established the auditory function of tympanic organs in Orthoptera, and of subsequent work upon these organs in Orthoptera, Lepidoptera and Hemiptera.

5. Experimental work indicating that tympanic organs may mediate a qualitative discrimination between different sounds is reviewed, and a tentative explanation of these observations is submitted.

6. Regen's experiments indicating the ability of gryllids to localize a sound source with considerable precision are described. It is shown that this ability might be expected from the physical structure of tympanic organs, and some direct evidence of their directional properties is presented.

7. The evidence of auditory powers in insects unprovided with tympanic organs is reviewed. It is shown that hair sensilla are in certain instances receptors for sounds of relatively low frequency, in contrast with tympanic organs which respond only to relatively high frequencies.

8. Other possible sound receptors are briefly discussed.

It is a pleasure to acknowledge my indebtedness to Dr A. D. Imms for his kindness in reading the manuscript of this review and for his friendly criticism.

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ERRATUM

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# THE FINE STRUCTURE OF BIOLOGICAL SYSTEMS

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(*Received* 13 June 1939)

CONTENTS



## I. INTRODUCTION

Had the influence of Darwin's theory been less powerful, the subjects to be discussed here would not perhaps have been forgotten for more than half a century. To-day the development of our knowledge of the molecular structure of certain classes of materials has made possible the rehabilitation and extension of ideas first developed by the botanist, Carl Nägeli, in the middle of the last century.

Nägeli studied the structure of plant cell walls and of starch grains, examining their optical properties (as revealed by the polarizing microscope), and also their behaviour (swelling or shrinking) in various media. Some of his views are expounded in the book which he wrote with Schwendener, *Das Mikroskop* (1877), and a valuable selection from his writings has been made by Frey (1928). To Nägeli we owe the term "micell".

## II. THE HISTORY OF THE MICELLAR THEORY

The term "micell" "denotes nothing more than a minute crystal of size far beyond the limits of resolution of the microscope" (Nägeli, 1879). Such particles were supposed to be the structural units of "starch grains, plant membranes and other organized structures, whether these are composed of protein-like, mucilaginous, elastic, horny or other substances" (Nägeli, 1879). The properties which Nägeli regarded as evidence of micellar structure were birefringence and the power of swelling. The birefringence ( ... an order-
liness of fine structure compara ... als. Since
the structures in question wer ... crystalline

particles, he postulated the existence of ultramicroscopic crystalline particles—the micells. Turning to the swelling phenomena, Nägeli pointed out that the particles composing a grain of starch, for example, do not pass into solution when the grain is placed in water, yet water is taken up and packed away within the grain. He concluded, therefore, that the grains are not built up immediately from the ultimate molecules, arranged in a continuous and regular manner, but that the first structural units are crystalline groups of molecules (micells), which in the imbibed condition are separated from each other by a layer of water.

Critics of Nägeli showed that birefringence may be induced in otherwise isotropic substances if these are placed under strain (in glass, for example). It was argued, therefore, that the observed birefringence of certain biological structures need not necessarily indicate an ordered structure. Even supposing that in many cases the microscopic objects observed were not under strain, it appeared difficult to reconcile the behaviour of birefringent cell components with that of birefringent crystals. It was observed, for example, that changing the refractive index of the medium in contact with the object sometimes changed its birefringence, and that in certain media the birefringence might fall to zero.

In 1912, however, Nägeli's ideas were much extended and amplified by Wiener's theoretical discussion of the optical properties of systems consisting of regularly arranged, anisodiametric, isotropic particles embedded in an isotropic medium of different refractive index. Wiener (1912) showed that if the particles are small in one diameter as compared with the wave-length of light, such a system will be birefringent, and the magnitude of the double refraction will vary with the refractive index of the medium bathing the particles. The theory was confirmed experimentally by the work of Ambronn and his collaborators (1916, 1926), and led to the prediction of various classes of birefringent structures. Two main types of birefringence were distinguished; one due to relatively coarse submicroscopic structure, *Formdoppelbrechung*, of which the commoner variety is *Stäbchendoppelbrechung* (rodlet birefringence), and the other due to the optical anistropy of the particles themselves, *Eigendoppelbrechung*, which (following Schmitt, 1939) will be referred to here as intrinsic birefringence. Structural birefringence varies with the refractive index of the surrounding medium; intrinsic birefringence is independent of such change. This statement presupposes that the liquids of varying refractive index in which birefringence is observed have no action on the micells, that they merely fill the intermicellar spaces.

While the detailed examination of plant tissues in the light of Wiener's theory was due chiefly to Ambronn and his pupil Frey-Wyssling, the extension of the work to animal cells and tissues—with which we are more particularly concerned here—has been due to Schmidt and his pupils. The greater part of the results of the study of the optical properties of animal structures described here is taken from Schmidt's work.

The second development of technique, which had led to the confirmation and extension of many of Nägeli's ideas, was the discovery by Laue (see Friedrich, 1922) of the diffraction of X-rays by crystals. The history of this development lies

within the last twenty years. From the results of X-ray analysis, evidence was brought forward by Scherrer (see Zsigmondy, 1927) that the particles in a gold sol give rise to an X-ray diffraction pattern, just as does a macroscopic crystal of gold (whence Zsigmondy's use of the term "micell" to describe the particles of the disperse phase in a colloidal solution). Finally, at Ambronn's suggestion (see Frey, 1928), Scherrer examined the X-ray diffraction patterns of plant fibres and found that the beam was diffracted as by a crystalline substance. In this way the crystalline nature of the cellulose micell was established. The analysis of the structure of cellulose fibres was extended in 1926 when Sponsler & Dore suggested the first three-dimensional model representing the structure and arrangement of the cellulose molecules within the micell. This model, which was unsatisfactory from several points of view, was replaced by that of Meyer & Mark (1928*a*).

Since that time our knowledge of micellar organization has been made more precise, largely as a result of the development of methods of X-ray analysis. We know now something of the shape and size of the micells in many natural fibres and membranes. In certain cases the arrangement of the molecules within the micell and of the atoms within the molecules is known with a fair measure of certainty. This is true of cellulose fibres such as ramie (from China-grass, *Boehmeria nivea*), of silk, and, to a slightly less extent, of chitin. It is to Meyer & Mark (1928*a*, *b*, 1929), who put forward the first satisfactory models of cellulose and silk, that the concept of "chain molecules" is due, a concept which has been of great importance for the amplification of Nägeli's original picture of micellar organization.

The term "chain molecule" is used to denote molecules which are composed of atoms or groups of atoms united in a linear series by primary valences. The properties of such molecules are, as we shall see, of profound importance for the explanation of the peculiar and characteristic properties of organized substance.

Gerngross *et al.* (1930) were among the first to suggest that the micells are incompletely separated one from another; that is, they are not completely discrete crystallites, as conceived by Nägeli. From their work on the fine structure of the gelatin gel, they concluded that the chain molecules may be considerably longer than the crystallites themselves, and may participate in the formation of several micells (Fig. 1). In other words, a gelatin gel may be pictured as an intricate tangle of chain molecules bundled together in certain regions to form minute crystal-like aggregates. The gel contains an amorphous "liquid" component—the tangled chain molecules—and a more "solid", "crystalline" component—the micells; the same chain molecules may participate in both components.

In 1937 Frey-Wyssling was able to extend the picture of micellar constitution by determining the size of the intermicellar (submicroscopic) spaces in ramie fibres (cellulose). This was done by impregnating the fibres with gold and estimating the size of the gold particles between the cellulose micells from the width of the gold lines in the X-ray diffraction pattern of the impregnated fibres. He found this system of spaces to form a continuous network throughout the fibre. The spaces were of about the same dimensions as the cellulose micells—50–60 A wide and 500–600 A long (1937*a*, *b*).

The third and most recently developed line of attack has arisen from the study of the mechanical properties of natural and synthetic high polymers, that is, of compounds of very high molecular weight built up from units of low molecular weight united by primary valences. Such work has been developed by K. H. Meyer and his collaborators and extended to the study of tendon and elastic ligament (Meyer & Ferri, 1936), muscle (Meyer & Picken, 1937) and elastoidin (Picken, 1937). The value of this line of attack is that it provides information about the shape and properties of molecules which are not necessarily united to form micells; that is, we may obtain insight into the structure of amorphous materials from a study of their mechanical properties and in particular their elastic and

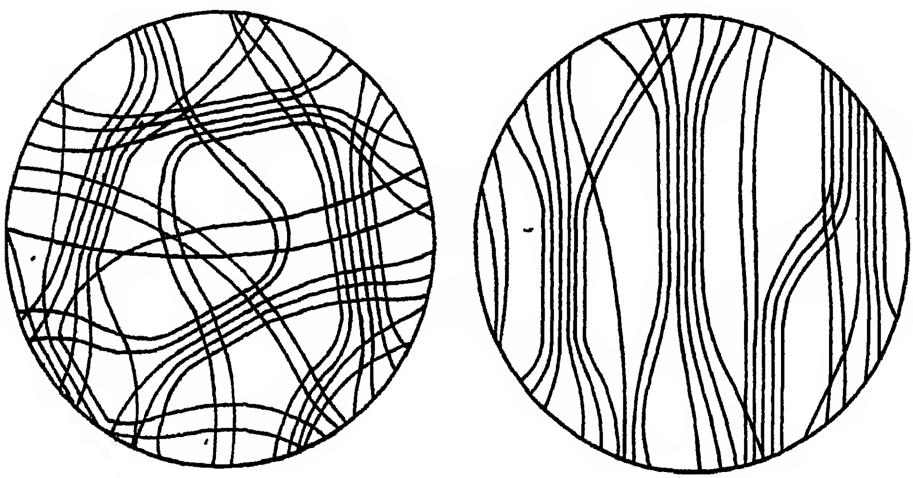


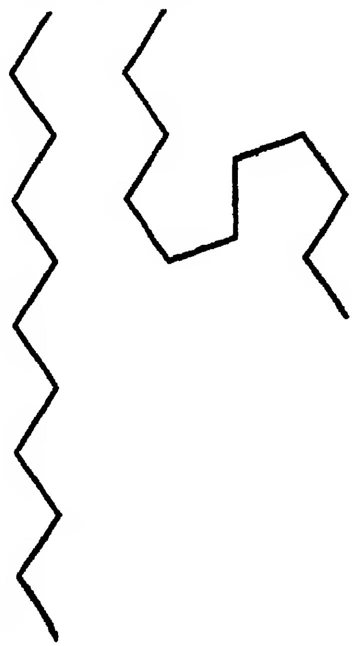

Fig. 1. A. Diagram showing the association of portions of long chain molecules to form parallel bundles ("crystallites"). B. Stretching leads to the parallel orientation of the molecules and "crystallites". (After Gerngross *et al.* 1930; Frey-Wyssling, 1939.)

thermoelastic properties. It is now realized that the presence of long flexible chain molecules is responsible for the high reversible extensibility shown by many natural products—rubber, muscle, elastin, and collagen and elastoidin above a certain temperature—as well as synthetic, rubber-like, organic substances such as polyvinyl acetate, polyvinyl alcohol, polystyrene, etc., and inorganic substances in the "elastic" condition—sulphur, selenium, polyphosphonitrilic chloride, etc. In the rubber-like state such molecules tend to assume highly probable configurations. Stretching leads to the straightening and orientation of the flexible chains (Figs. 1 B, 2); their configurations become less probable (in the thermodynamic sense). When the deforming force is removed, thermal agitation leads to the chains assuming highly probable configurations once more and the object contracts. If such a rubber-like object is stretched and the length kept constant, the elastic force exerted increases as the temperature rises, diminishes as the temperature falls.

In a normal elastic solid, deformation results in the displacement of atoms or groups of atoms from positions of minimal potential energy; the internal energy

increases on stretching, only to diminish when tension is removed, and the object contracts. Stretched at constant length, the elastic force exerted by such an object (as, for example, by a steel spring) diminishes on warming, and increases on cooling.

A rubber-like substance behaves in a fundamentally different way; it may be stretched without any change in internal energy taking place—the chains are simply straightened out (Meyer & Ferri, 1935), or the internal energy may even diminish on stretching, if, as a result of orientation, the chains are brought into alignment such that they form crystallites with the liberation of heat of crystallization. The presence of rubber-like high reversible extensibility in an object enables us to say at once that its elastic properties are due to the presence of long flexible chain molecules; the detailed study of these elastic properties can take us even further towards an understanding of its molecular structure.



*a* *b*

Fig. 2. Diagram illustrating the conversion of a stretched carbon atom chain to a more probable configuration, by rotation of the links without distortion of valency angles and therefore without increase in internal energy. Note that while there is only one stretched configuration, many twisted forms are possible, that is, the stretched configuration is the least probable.

In the light of present knowledge it seems desirable to re-define the term "micell"; for while there are certain types of micell—such as the crystallites of cellulose and chitin—which correspond to Nägeli's definition, there are others to which the adjective "crystalline" is scarcely applicable. It is proposed, therefore, to define a micell as an anisodiametric particle possessing some measure of internal structural regularity. Such particles are usually built up from chain molecules arranged in a more or less orderly manner. This may be little more than an approximate parallelism of the chains (as in the simplest type of liquid crystal) or the structure may be truly crystalline in the usually accepted sense of the word.

It is doubtful how far the process by which such molecular aggregates come into being is to be compared with the crystallization of compounds of low molecular weight. Growth of the chains may occur during their association to form crystallites, and existing crystallites may increase in length as a result of growth of the individual chains; that is to say, chemical synthesis may be involved. On the other hand, chain molecules may under certain circumstances be brought to approximate parallelism (as a result of deformation by external forces) without any considerable change in internal energy, taking place, that is, in the absence of true crystallization. These considerations must be borne in mind in reading what follows.

## III. LINEAR AND LAMINAR FINE STRUCTURE

In the presentation of the material to be discussed in this review, the classification proposed by Herzog (1932) will be used as a basis on which to group the types of structure to be described. Herzog drew attention to the importance of two main types of molecular aggregate in biological systems—linear (or fibrous) and laminar structures. From a consideration of the types of structure found in skeletal materials (in the widest sense), Herzog showed that these may be regarded as built up from a series of structural elements of increasing size and complexity. The primary structural units are linear macromolecules (chain molecules) which associate to form secondary elements—linear crystallites, which are again bundled together to give primitive fibres. These last lie on the limit of resolution of the microscope and were regarded by Herzog as the "primitive biostructures" serving as the structural units for still higher types of organization, that is, for macrofibres, in which the primitive fibres are bundled together to form a linear structure, and for laminar structures, in which the primitive fibres lie side by side forming a sheet one fibre thick. The terms "fibrous" and "laminar" will be used here to denote the way in which the minute crystallites composing a particular structure are arranged; that is, either to form fibres in which one particular axis of each crystallite is parallel to that axis in every other crystallite (fibre structure in Polanyi's sense; see Herzog *et al.* 1920), or to form sheets in which the long axes of the micells lie in the plane of the sheet, but need not necessarily be parallel to each other (so that all laminar structures are not necessarily dissociable into primitive fibres in Herzog's sense). It will be seen that the term "fibril" is used in these pages as the equivalent of Herzog's primitive fibre.

The wide range of structures to be discussed—from cilia and chromosomes to bone and muscle—necessitates a somewhat unusual grouping of the material, resembling to some extent the system proposed by Heidenhain (1923, 1929). All parts of cells (cell components in Russell's sense, 1930) will be classed as subcellular: membranes, cilia, mitotic spindles, etc. Fibres and membranes formed extracellularly will be described as extracellular: fibres of elastoidin, elastin and collagen, spicules of various kinds, membranes or fibres of cellulose or chitin. Finally, a group of fibres and membranes of multicellular origin, whose macroscopic form is obviously related to their micellar and molecular organization, will be classed as supracellular: hair, horn, bone, scales, muscles, etc.

The scope of this review will be limited almost entirely to animal structures, not because the botanical material is less abundant (it is perhaps almost richer) but in order that there may be space for general discussion of the results reviewed. An excellent account of the botanical significance of the chain molecule structure of cellulose will be found in a review by Preston (1939).[1] No attempt will be made to describe the large body of work on the structure of the various kinds of spicules which occur in many groups and which have received attention in recent years (see Schmidt, 1924*a*). Those in search of an introduction to the phenomena of birefringence may refer to recent reviews by Schmitt & Bear (1939),[1] and

[1] *Biological Reviews.*

Preston (1939),[1] to Schmidt (1934, 1937*b*), to Frey-Wyssling (1939) or to Ambronn & Frey (1926). An account of certain aspects of fibre structure is given by Astbury (1933), and a general account of the whole field of high polymer studies will be found in Meyer & Mark (1930, 1939).

## IV. SUBCELLULAR STRUCTURES

### (1) Fibrous structures

#### (*a*) *Protein fibres from the sap of virus-infected plants*

From the sap of tobacco or tomato plants infected with tobacco mosaic virus, needle-like crystals, about 40 $\mu$ long, can be isolated. This "crystalline" protein, which has the character of a nucleoprotein, possesses the properties of the virus, and no process of purification has yet resulted in the separation of virus activity from the protein (Stanley, 1936; Bawden *et al.* 1936; see also the recent review by McFarlane, 1939[1]). Concentrated solutions of virus proteins have been observed to separate into two layers, of which the lower is spontaneously birefringent and the upper becomes birefringent when flowing. This tendency of the virus protein particles to associate to form birefringent aggregates is of considerable biological interest in view of the suggested parallel between viruses and genes (see discussion in Gardiner, 1931). Bernal & Fankuchen (1937) have pointed out that the "crystallinity" of the protein needles is of a special kind. The needles show a regular repetition of their fundamental units in two dimensions only, that is, in the plane at right angles to the long axis; along the needle axis no such regularity is observed. The fibres correspond, therefore, to one of the theoretical types of liquid crystal (compare also with the structure of polyvinyl acetate, Misch & Picken, 1937). From the X-ray diffraction pattern it appears that the particles are packed in a regular hexagonal manner, each particle being about 150 A wide. The X-ray pattern also shows that the particles themselves possess a regular internal structure in three dimensions, a structure which is similar for three strains and two varieties of virus which have been examined. On the other hand, the pattern due to the regular spacing of the particles is slightly different in each case, and for this reason Bernal & Fankuchen (1937) have suggested the possibility of classifying the viruses on the basis of the X-ray patterns from virus protein fibres. If we accept the view that the virus protein is identical with the virus (as all the evidence suggests), we have here anisodiametric organisms, of the same order of size as a large protein molecule, which are capable of being packed together to form rudimentary crystals.

#### (*b*) *Chromosomes*

A stimulating attempt "to interpret in molecular terms the postulates of genetics and the nature of the gene" has been made by Wrinch (1936), whose arguments for an ordered micellar structure of chromosomes have been much strengthened

[1] *Biological Reviews.*

by the recent researches of Schmidt and his collaborators on the optical properties of chromatin in a great range of nuclei. Engelmann (1875) drew attention to the optical anisotropy of both head and tail in sperm from winter frogs; he noted that the birefringence of the head was negative and that of the tail positive with respect to the long axis. Brandt (1885) observed birefringence in nuclei during the later stages of isospore formation in certain Radiolaria (in particular *Myxosphaera coerulea*); the nuclei appeared as strongly birefringent, wedge-shaped blocks forming a sphere bounded by the surface of the central capsule. Squashing the capsules or immersion in glycerine led to disappearance of the birefringence, but balsam preparations were almost as strongly birefringent as in life. Schmidt (1932*a*, *b*), repeating these observations on radiolarian nuclei, was able to show that the birefringence of each of the obtusely conical nuclei arranged over the surface of the central capsule was negative with respect to the axis of the cone. Such nuclei showed traces of fibrillar structure parallel to the axis of the cone, but it was not possible to refer the birefringence to definite chromosomes. He observed, however, that fibrillar strands of chromatin, obtained from these nuclei by shearing pressure on the coverslip, were negatively birefringent with respect to the fibre axis, and retained their birefringence in balsam. Treatment with sea water led to the disappearance of birefringence, which reappeared on dehydration in alcohol. The birefringence visible in balsam is intrinsic birefringence for, since the threads are invisible in ordinary light when embedded in balsam, their refractive index must approximate to that of balsam. On swelling in water the disappearance of birefringence may be due to compensation of the negative intrinsic component of the birefringence by positive structural birefringence, or to the disorientation of the micells during imbibition.

In metazoa, Schmidt (1928*b*, 1932*a*) was able to show birefringence in nuclei of the gut cells in ophioplutei and echinoplutei fixed with absolute alcohol and mounted in balsam. Later (1936*a*, *b*) it was possible to demonstrate the birefringence of the chromosomes of cleaving eggs of *Psammechinus miliaris*. The chromosomes are positively birefringent with respect to their long axis. This means that the negative birefringence of the chromatin micells is overcompensated by positive structural birefringence, assuming (as is probable) that spiralization does not occur in these chromosomes.

Schmidt has pointed out that Wrinch's assumption, that the chromosomes are bundles of polypeptide chains held together by transversely disposed nucleic acid molecules, is not reconcilable with the observed birefringence of chromosomes and of nucleic acid. He finds that the chromomeres of the salivary-gland chromosomes of *Chironomus* are negatively birefringent with respect to the long axis of the chromosome (Schmidt, 1938*c*). Since fibres of thymonucleic acid jelly are also negatively birefringent, and since solutions of nucleic acid exhibit negative birefringence of flow (Signer *et al.* 1938), it is to be concluded that the molecules of thymonucleic acid are negatively birefringent with respect to their long axis and, therefore, that the molecules of nucleic acid in the chromomeres lie parallel to the polypeptide chains. This picture is supported by the findings of Astbury & Bell (1938), who

observed an identity period of 3·34 Å along the fibre axis in X-ray diagrams from stretched films of thymonucleic acid. This period, which corresponds to the spacing of the nucleotides, is almost the same as the length of a fully stretched polypeptide residue. They suggested that this may make possible the association of polypeptide and nucleotide chains. This suggestion has been confirmed by their work on a fibrous compound of clupein and thymonucleic acid (Astbury & Bell, 1938*b*) in which they have shown that the nucleotides fit directly on to the side chains of a fully extended polypeptide chain. The optical properties of this compound are analogous to those of straight chromosomes.

In extension of the work on chromatin, Schmidt (1937*b*) has examined the optical properties of strips of sodium $\alpha$-thymonucleate jelly. On stretching previously isotropic strips, these become strongly negatively birefringent with respect to the long axis. Since stretching will lead to the orientation of anisodiametric particles with their longest axis parallel to the direction of stretching, the birefringence observed cannot be structural birefringence (which would be positive) and is presumably, therefore, negative intrinsic birefringence. Suitably prepared strips of stretched jelly, dehydrated with absolute alcohol, show the silky sheen of tendon, suggestive of fibrous structure; their fibrous character is confirmed by microscopic examination. Certain parts of the strips may show weak positive birefringence (structural birefringence) which disappears when the material is immersed in balsam ($n_D$ balsam $= n_{\|}$ sodium $\alpha$-thymonucleate). From a variety of experiments Schmidt concludes that sodium $\alpha$-thymonucleate jelly consists of anisodiametric particles which may be orientated by stretching and show a marked tendency to adopt fibrillar arrangement. The fibrils so formed shorten and thicken when allowed to swell or when treated with certain reagents, and their birefringence diminishes or disappears under such conditions.

Schmidt emphasizes the parallel between the high elastic extensibility of the sodium $\alpha$-thymonucleate gel and that of the chromatin threads of spermatocytes (Chambers, 1924), and of the nucleus of the red blood corpuscles of Amphibia (Seifriz, 1927), and concludes that the properties of the nucleic acid jelly make it certain that many of the optical and mechanical properties of the chromatin of the nucleus are due to its nucleic acid component. The parallel between the behaviour of chromosomes and the tendency of fibrillar structures to split longitudinally, to swell laterally and to contract, is immediately apparent.

Schmidt's study of the chromatin of the sperm head will be discussed later.

### (*c*) *Asters and spindles*

Schmidt (1936*a*, *b*) has demonstrated the positive birefringence of spindle and asters in living cleaving eggs of *Psammechinus miliaris*, each aster showing a positive spherite cross. Since the aster fibres end freely in the cytoplasm, it is difficult to imagine that the birefringence is due to tension. On squashing the eggs the spindle retains its form as a solid body and only loses its birefringence when it begins to swell under the action of the sea water. The conception of the spindle as a micellar aggregate of the fibrillar type (which its optical properties suggest) is supported by

its anisotropic shrinkage on dehydration. Bělař (1929) showed that the width of the spindle diminishes considerably on dehydration while its length remains unchanged. This occurs in all fibrillar structures in which water is packed between orientated anisodiametric particles.

### (d) *Contractile fibrils*

Engelmann (1875) found all contractile protein fibrils which he examined to be positively birefringent with respect to the long axis, and he concluded that all such fibrils possess an orderly internal structure. This conclusion is confirmed and extended to-day as a result of work on one particular type of contractile fibril—that which is present in muscle fibres. Since the observations of Brücke (1848) on the birefringence of striped muscle, an enormous literature has grown up, which it is impossible and unnecessary to review here. Reference may be made to the books of Schmidt (1924 *a*, 1937 *b*), and to papers by Fischer (1936), Weber (1933), von Muralt (1933) and Boehm (1934). Engelmann's evidence for the orderly structure of contractile fibres has been added to in several different ways: (1) by an analysis of the form and micellar birefringence in resting and contracting muscle (see Schmidt, 1937 *b*; von Muralt, 1932); (2) by an analysis of the birefringence of myosin fibrils and a study of the birefringence of flow of myosin solutions (von Muralt & Edsall, 1930); (3) by the study of the X-ray diffraction patterns of living muscles—both resting and contracting (Boehm, 1931; Meyer & Picken, 1937; Astbury, 1938), and of dried muscles and myosin films and filaments (Boehm & Weber, 1932; Astbury *et al.* 1935 *a*, *b*, 1936); and (4) by the study of the elastic properties and in particular of the thermo-elastic properties of living, resting muscles and their comparison with the properties of rubber-like high polymers, both natural and synthetic (see Meyer & Picken, 1937, for complete references).

(i) *Muscle fibrils.*

The two ends of a muscle fibril are held together by a loose three-dimensional network of flexible, long chain, polypeptide molecules in the meshes of which lie similar free chains (Meyer & Picken, 1937). The chains (that is, the myosin molecules) are associated in certain regions to form minute "crystallites"—liquid crystals possessing a rudimentary three-dimensional regularity (from X-ray evidence, Boehm, 1931; Meyer & Picken, 1937; and from the thermoelastic properties, Meyer & Picken, 1937). A certain fraction of the micells are orientated with their long axes parallel to the fibril axis (X-ray and birefringence data), and the orientation can be slightly increased by stretching the muscle as is shown by X-ray evidence (Meyer & Picken, 1937) and birefringence data. Fischer (1936) showed that a slight increase in structural birefringence takes place on stretching the muscle, but it appears that the micells are mostly parallel to the fibril axis even in the resting, unstretched muscle. The study of the birefringence of muscle fibrils by Stübel (1923), Weber (1933), and Fischer (1936), has indicated that this is in part structural birefringence and in part positive intrinsic birefringence. Since the micells are mostly parallel to

the fibril axis, the muscle fibril is to be regarded as an ideal *Mischkörper* (Wiener), in which the regularity of the micellar arrangement is already maximal.

The chain network is responsible for the rubber-like elastic and thermoelastic properties of resting muscle—properties which it shares not only with rubber but with many other high polymers showing high reversible extensibility (Meyer & Picken, 1937).

X-ray studies of living muscles have shown that there is a periodic structure in the plane at right angles to the fibril axis repeated at intervals of 10 A (Boehm, 1931; Meyer & Picken, 1937), and one along the fibril axis repeated at intervals of *c.* 5 A. The transverse spacing may be compared with that shown by keratin (Astbury & Dickinson, 1935 *a*, *b*), and may perhaps be identified with the average length of the side chains between neighbouring protein chains. The resemblances between dried muscles, myosin films and fibres, and keratin fibres will be discussed later.

It is clear that the myosin chains are continuous from one end of the fibril to the other, even though the fibril may show striation and appear to be divided into alternating segments of isotropic and anisotropic material. The view most generally held (see von Muralt, 1933) is that the isotropic segments are regions in which the micells are orientated entirely at random, while in the anisotropic regions they are for the most part parallel to the fibril axis. In any case, the molecular network must be continuous. It is possible to induce striped fibrils to lose their striation without the fibril ceasing to be excitable (Liang, 1936), and the conversion of smooth fibres to striped is well known in development, as is the converse change in pathological conditions (see Schmidt, 1937*b*). Bernal (1937) has suggested that the striation is comparable to the layering of concentrated solutions of anisotropic particles observed in capillary tubes (see, for example, van Iterson, 1934), and may be interpreted as the most efficient way of packing flexible chain molecules in narrow tubes with maximum utilization of the space available. We have to account, however, for the fact that the whole fibre is striated—the striations of all the fibrils occur at the same level—and that the fibres appear to be held together at the isotropic layers (Schmidt, 1937 *b*). It seems not impossible that striation within the fibril is due to an effect such as Bernal suggests, and that outside the fibril a network of some kind holds the fibrils together at the isotropic layers (Liang, 1936).

Various lines of evidence point to the conclusion that the contraction of a muscle fibril is associated with a change in the configuration of the myosin chains, such that the structure of the contracted muscle is less orientated than that of the resting muscle. Many workers have observed that the birefringence diminishes during isotonic contraction (see Schmidt, 1937 *b*), and von Muralt (1932) demonstrated a diminution in birefringence of the sartorious muscle of the frog during isometric contraction. The X-ray diffraction pattern shows a corresponding disappearance of orientation during isotonic contraction (Boehm, 1931; Meyer & Picken, 1937; but according to Boehm a slight enhancement of the degree of orientation during isometric contraction). Astbury (1938) finds that the changes in the X-ray diagram during isotonic contraction resemble those observed in the supercontraction of keratin (see p. 154). The disorientation which occurs is disproportionately small in

relation to the contraction observed. Fischer (1936) offers reasons for believing that the contraction depends essentially on internal rearrangement of the micells rather than on disorientation of the micells as such.

There are grounds then for believing that the change in shape of a muscle fibril on stimulation is but the sum of changes taking place in the myosin molecules. Fibrillar structure serves to make visible microscopically—and eventually macroscopically—the effects of change in molecular configuration of the constituent molecules. With this scheme in mind we shall now examine the evidence for a similar structure in other contractile fibrils.

### (ii) *Myonemes.*

Engelmann (1875) estimated the brilliance of the stalk myoneme of *Zoothamnium* viewed between crossed nicols as at least as great as that of vertebrate or insect muscle fibres in balsam preparations. The fibrils of the myoneme were observed to be positively birefringent (with respect to the long axis) and surrounded by isotropic protoplasm. Engelmann noted the shortening and thickening of the fibrils when the myoneme contracted. Watching the formation of the stalk from granules of protoplasm he found that development of the myoneme begins at the boundary of stalk and body as soon as the stalk has reached a length of *c.* 0·4—0·6 mm. From the first moment of its appearance the myoneme is both contractile and birefringent. Mackinnon & Vlès (1908 *a*, *b*) re-investigated the myonemes of the stalk of *Carchesium*, and showed that, on immersion in liquids of widely differing refractive index, the birefringence persisted; in the light of Wiener's theory this means that the myoneme possesses considerable intrinsic birefringence. Among other ciliates examined by Engelmann may be mentioned *Lacrymaria olor*, the "neck" of which is remarkably contractile; viewed between crossed nicols with a gypsum plate inserted the neck appeared violet (addition position) or orange (subtraction position).

### (iii) *Pseudopodia.*

The positive uniaxial birefringence of the axopodia of the heliozoan *Actinosphaerium* was described by Engelmann (1875), who also observed between crossed nicols the penetration of the axial filament into the body. On withdrawal of the pseudopodia (as a result of electrical stimulation, for example) the birefringence disappears. Mackinnon (1909) confirmed these observations and also detected a faint gleam from the rest of the protoplasm when viewed between crossed nicols. She showed that the birefringence of the axial filament disappeared if the medium bathing the axopodia were clove oil ($n_D = 1{\cdot}53$) or creosote ($n_D = 1{\cdot}54$). In liquids of lower refractive index, such as water ($n_D = 1{\cdot}33$), or of higher refractive index (monobromnaphthaline, $n_D = 1{\cdot}66$), the birefringence was marked. These results indicate that the axial filament possesses structural birefringence, and may be supposed to consist of anisodiametric micells, the long axes of which lie parallel to the axis of the axial filament (since the birefringence is positive with respect to the long axis of the pseudopodium).

Schultz (1915) observed that the axial region of the pseudopodia of the foraminiferan *Astrorhiza limicola* was uniaxially birefringent, the optical axis lying in the direction in which shortening occurs on contraction. Schmidt (1937*a*) recorded weak birefringence of the pseudopodia in living specimens and permanent preparations (in balsam) of the radiolarian *Thalassicolla*, and in the foraminiferan *Miliola* he was able to show appreciable birefringence of the pseudopodia bundles and to photograph single pseudopodia between crossed nicols, using a 1/16 W.L. mica plate in the addition or subtraction position to enhance or compensate the birefringence of the object. In this way the positive birefringence of the single filopodium was established.

(iv) *Cilia.*

Vlès (1908), Mackinnon & Vlès (1908*a*, *b*) and Vlès (1911) investigated the birefringence of the ciliated epithelium of *Mytilus* gills, the cilia of *Stentor* and *Vorticella* and the ciliated plates of ctenophores, in media of differing refractive indices. They found that the birefringence disappears in media of $n_D = 1{\cdot}51{-}1{\cdot}53$, but is marked in media of lower or higher refractive index (this could be observed in the same preparation in different media). Schmidt doubts the entire absence of intrinsic birefringence which these results show. He observes in preparations of the ciliated combs of *Beroë*, fixed in alcohol and mounted in balsam ($n_D = 1{\cdot}53{-}1{\cdot}54$), a faint but definite positive birefringence, whereas Mackinnon & Vlès found no birefringence in liquids of this refractive index.

The birefringence of the fresh, ciliated combs of *Beroë* undergoes interesting changes as a result of the precipitation of lipoids, and their adsorption on the cilia when the combs are treated with certain reagents (Göthlin, 1913; Schmidt, 1925*b*). In the presence of alcohol, for example, the first effect is a change in sign of the birefringence from positive (with respect to the long axis of the plate) to negative, as a result of the orientated adsorption of the precipitated lipoid particles, which are themselves anisodiametric and show birefringence (negative with respect to the longest axis). Further treatment with alcohol leads to the solution of the lipoid material and the birefringence becomes positive once more.

Birefringence of the tails of sperm was first described by Engelmann (1875) in sperm from winter frogs. Schmidt (1937*b*) has published striking photographs showing the birefringence of rat sperm tails. Mackinnon & Vlès (1908*a*, *b*) studied the relation between birefringence and refractive index of the medium in sperm tails of *Triton cristatus*, and found almost complete disappearance of the birefringence in media of $n_D = 1{\cdot}51{-}1{\cdot}54$. Schmidt (1937*b*) showed, however, that the tails of alcohol-fixed sperm (from the Fire Salamander) mounted in balsam of refractive index approximating to that of the material, were positively birefringent with respect to their long axis. He concluded that the sperm tail possesses both form and intrinsic birefringence. The longitudinal splitting of the sperm tail after maceration (Ballowitz, 1889) offers additional evidence for its submicroscopic fibrillar structure.

### (2) Laminar structures

#### (*a*) *Surface membranes of animal cells*

This subject has been discussed recently by Harvey & Danielli (1938) and by Schmitt & Bear (1939),[1] and will not be considered here. It is desired to draw attention to the possible mechanical importance of the protein laminae in cell membranes. Meyer (1937) pointed out the significance—in the light of present views on the molecular structure of natural and synthetic rubber-like polymers—of the high reversible extensibility of red blood corpuscles (affecting both contents and membrane), which was demonstrated by Seifriz (1927). Such elastic properties are not explicable in terms of sheets of lipoid molecules. It is necessary to suppose that flexible, long-chain molecules are present in the surface, and that these are united at certain points to form a loose network; for since the surface can be drawn out to a filament ten times the diameter of the cell in length and yet return to its original configuration, it is presumably cross-linked to some extent (like vulcanized rubber). The conception of the protein laminae as cross-linked films would help to explain the preservation of cell shape; pursuing the analogy with rubber, we may envisage the surface protein laminae as "vulcanized" *in situ* (see Meyer & Mark, 1939), so that the surface tends to preserve its specific shape, and yet has elastic properties which, at first sight, simulate those of the surface of an oil drop in water.

#### (*b*) *Rods and cones*

Valentin (1861) first described the positive uniaxial birefringence of the outer portion of the rods and cones in the retina of the frog, the optical axis being parallel to the longitudinal axis of the structure. Dimmer (1894) showed that lipoid solvents reverse the sign of the birefringence (human retina). Schmidt (1928*a*) observed that the disappearance of positive birefringence coincides with the disappearance of the fatty sheen from the rods. These observations, which pointed to some correlation between positive birefringence and the orientated arrangement of lipoid material, were extended by Schmidt (1935) and, as a result, it was found that a close parallel exists between the optical properties of the myelin sheath of nerve and those of the retinal rods and cones. In the case of the myelin sheath it is known (from the work of Schmidt, 1936*c*; Schmitt & Bear, 1939) that the optical axis of the positively birefringent sheath is radial, and that the myelin molecules lie with their long axes in this direction. Since the optical axis of the outer portion of the rods and cones is parallel to the long axis of these structures, it is to be supposed that the long axes of the lipoid molecules lie parallel to the rod or cone axis.

As in the myelin sheath, where the double layers of lipoid molecules (Schmitt & Bear, 1939) are supported by non-lipoid (protein) layers, so in the rods and cones the double sheets of lipoid molecules are separated by layers of non-lipoid nature. The presence of these layers is shown by the tendency of the structure to break up into minute leaflets, the plane of which is at right angles to the axis. When the lipoids are dissolved out, the sign of the birefringence changes from positive to negative.

[1] *Biological Reviews.*

This means that the molecules in the non-lipoid, protein laminae lie with their long axes at right angles to the rod axis. The orientation of the molecules in the laminae is shown by the lengthening of the rods on warming or on treatment with swelling agents such as potassium hydroxide or dilute mineral acids. Under similar conditions, protein fibres, in which the chain molecules run parallel to the fibre axis, increase in diameter. Thus the protein molecules in the non-lipoid layers of the rods and cones lie at right angles to the axis of the structure (see Fig. 3). Schmidt's experiments show that the negative birefringence of the rods after freeing from lipoids is structural birefringence.

This picture of the rods and cones as voltaic pile-like structures, consisting of alternate sheets of lipoid and protein, has been supplemented by a consideration of the structural implications of the observed natural and artificial dichroism of these

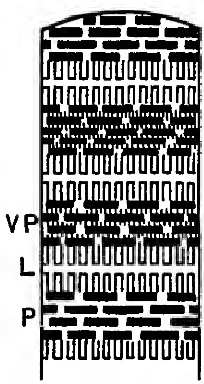



Fig. 3.

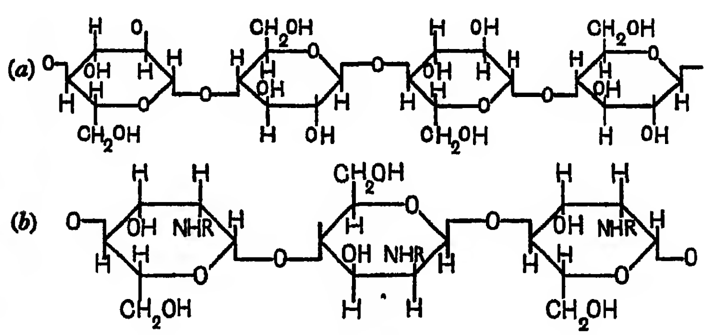



Fig. 4.

Fig. 3. Diagram showing the structure of the outer portion of the rods and cones (from Schmidt, 1937*b*). *L* = lipoid layer; *P* = protein layer; *VP* = visual purple adsorbed in an orientated manner on the protein layers.
Fig. 4. (*a*) Structure of cellulose (after Meyer & Mark, 1930). (*b*) Structure of chitin (after Meyer & Mark, 1930); R represents $CH_3CO$—.

structures. When fresh rods are examined in plane polarized light (without an analyser), they appear colourless if their long axes are parallel to the plane of vibration of the polarized beam, but are red if turned through 90°. As soon as the visual purple is bleached, the dichroic change is from colourless to yellow. The dichroism is that of a uniaxial crystal. On treating the retina with osmium tetroxide (which abolishes the positive birefringence due to the lipoids) the dichroism due to the visual purple persists, and Schmidt concludes, therefore, that the visual purple is not distributed in the lipoid phase. It may be supposed that the particles of visual purple are anisodiametric and are adsorbed in an orientated manner on the protein discs with their long axes parallel to the axis of the whole structure. Preparations of the retina impregnated with gold or silver are dichroic, the dichroism (with respect to the long axis of the rod) being opposite in sign to that shown by similarly impregnated protein fibres. This fact supports the conclusion already drawn that the protein molecules lie at right angles to the axes of the rods and cones. Schmidt

(1938*b*) has recently summarized his work on the protein-lipoid system in the outer portion of the rods and cones.

## V. EXTRACELLULAR STRUCTURES

When we come to consider those fibres and membranes which are formed outside the bodies of cells, we find these, in the animal kingdom at least, to consist for the most part of polypeptide or polysaccharide chains. The latter are generally supported by other highly polymerized substances, either aromatic in character (lignin, for example; see Frey-Wyssling, 1935) or possibly of protein origin in the case of chitinous structures such as the chaetae of annelids (Picken, 1939). On the whole, polysaccharide structures are very much more rigid than protein structures of the same dimensions. This may be ascribed in the first place to the more perfect crystallization of the polysaccharides (cellulose and chitin) as compared with the polypeptides (keratin, collagen, elastoidin, etc.); secondly, to the presence of substances occupying the intermicellar spaces. These substances include lignin, which occurs in association with cellulose, forming wood; silica, which fills the intermicellar spaces in the cell walls of grasses; *Inkrusten*, associated with chitin in the integument of arthropods, in the chaetae of annelids and elsewhere; and, in a case recently described by Jones *et al.* (1935), iron (in the form of oxide or carbonate) together with silica forms the hardening substance in the radula teeth of a limpet, *Patella athletica*. In the case of wood (Frey-Wyssling, 1935) the intermicellar spaces are so completely filled that the lignin skeleton, which remains after removing the cellulose, shows marked structural birefringence, owing to the regularity of the spaces left. Lignin itself is isotropic, and may be assumed to form a continuous three-dimensional network completely filling the spaces between the cellulose micells. A detailed study from the point of view of functional mechanics of the distribution and properties of fibres and membranes such as these has yet to be made.

The great tensile strength of the natural high polymers in general is due to their chain molecule structure. In the case of cellulose, the measured tensile strength is comparable with that of the finest steel, in spite of considerable irregularities in the texture of natural cellulose fibres (in this connexion see Meyer & Lotmar, 1935). The high tensile strength in the direction of the fibre axis is due, of course, to the fact that the cellulose units, from which the cellulose chains are built up (Fig. 4 (*a*)), are united by primary valences to form chains. In the case of an ideal fibre (that is, one in which the chains are continuous from end to end of the fibre), it is necessary to apply a force capable of separating atoms attached by primary valences in order to break the fibre, whereas in a crystal of an organic compound of low molecular weight (a crystal of glucose, for example), only intermolecular forces of attraction have to be overcome in order to separate one part from another. With respect to tension applied at right angles to the fibre axis, the fibre is much less resistant since, in the case of cellulose, the chains are united laterally by considerably weaker intermolecular crystal forces—hence the possibility of splitting such fibres longitudinally. It may be added that the most recent X-ray studies of cellulose (Meyer & Misch, 1937) suggest that the crystallites are extremely well-knit structures, and it is

probable that the tendency of the fibres to split longitudinally is due mainly to weakness in less highly orientated parts of the fibre where the lateral intermolecular forces are weakest.

### (1) Polypeptides

#### (a) *Fibrous structures*

(i) *Elastoidin*.

The transparent, homogeneous fibres (ceratotrichia) supporting the fin membranes of elasmobranchs and occurring as "actinotrichia" in the fins of all embryonic Teleostomes (Goodrich, 1930), have been studied by Schmidt (1924*b*, 1925*a*, 1928*a*, 1932*c*) and by Fauré-Fremiet and his collaborators (1936*a–e*, 1937*a*, *b*). Schmidt showed that the fibres are positively birefringent and uniaxial and that both structural and intrinsic components of the birefringence are present. He observed, moreover, that the sign of the birefringence is reversed when the fibre contracts on heating in water. These observations were confirmed and extended by Fauré-Fremiet & Woelfflin (1936*c*), who demonstrated that the micellar birefringence of the normal fibre is relatively large compared with the structural component. On heating in water to a temperature of about 62° C., the fibres shorten to about one third of their original length (Fauré-Fremiet & Woelfflin, 1936*d*), and at this temperature are rubber-like, showing high reversible extensibility; in this condition the substance has been referred to by Fauré-Fremiet as elastoidin II in contrast to the normal fibre, elastoidin I. On cooling, the contracted fibre relaxes and approaches (but never reaches) its original length. Under tension the fibre may be brought back to its original length, but since the tension necessary to do this is very near the breaking limit of the fibres, this operation is rarely successful. The X-ray study of elastoidin fibres (Astbury & Lomax, 1935) shows them to possess a typical fibre structure (that is, the crystallites all have one axis orientated in the direction of the fibre axis) and indicates that this protein belongs to the collagen group. Elastoidin II, on the other hand, is amorphous at or above 62° C., but below this temperature the X-ray pattern shows the original "crystalline" pattern of elastoidin I superimposed on the "amorphous" diagram of elastoidin II (Champetier & Fauré-Fremiet, 1937). The conversion of elastoidin I to elastoidin II may thus be regarded as the fusion of a "crystalline" structure, and may be compared with similar processes in many other high polymers. A piece of rubber, for example, may be stretched until it shows a "crystal" pattern and then frozen in this condition. On warming, the stretched rubber suddenly contracts and becomes once more reversibly extensible and amorphous. Since elastoidin II below 62° C. possesses a partially crystalline structure, it was predicted from thermodynamic considerations (see, for example, Meyer & Ferri, 1936), that the curve relating the elastic force exerted by a fibre stretched at constant length to the absolute temperature would have a certain slope. The study of the thermoelastic properties of elastoidin fibres (Picken, 1937) showed that this prediction was fulfilled. All the facts recorded here point to the similarity in molecular structure between elastoidin and other high polymers, and it seems clear that the contraction of the fibres on heating in water

is due to the liberation, from a state of crystal-like association, of long chain molecules which, as a result of thermal agitation, assume highly probable configurations (see Fig. 2), with the result that the whole fibre shortens.

In contrast to collagen fibres, elastoidin does not dissolve to form gelatin after "melting", nor do contracted fibres show appreciable relaxation—that is, if the fibre is suddenly stretched and its length thereafter kept constant, the elastic force exerted undergoes but little change in time (at a temperature of 62° C.). This behaviour signifies (1) that the flexible long-chain molecules of elastoidin II are united by cross-linkages to form a loose three-dimensional network, and (2) that the netting of the chains is very complete, for, since relaxation is slight, there can be but few chains present which do not form part of the network. The "crystallization" of the chains on cooling is not prevented by the presence of cross-linkages.

(ii) *Collagen.*

A very close parallel exists between the properties of elastoidin and those of collagen fibres (white fibres of connective tissue and tendon): the latter are also normally inextensible and "crystalline", but on heating to *c.* 65° C. they contract and are reversibly extensible at this temperature. If the melted fibres are stretched and cooled in ice-water they no longer contract when the tension is removed (compare their behaviour in this respect with that of other high polymers cooled under tension). The contracted fibres "flow" on stretching (like unvulcanized rubber) and rapidly dissolve in hot water to form gelatin; this implies that the chain molecules of collagen are not linked to one another as are those of elastoidin. Treatment of the fresh fibres with formaldehyde leads to the formation of bridges between the chains (Meyer & Ferri, 1936), and collagen fibres so treated (pieces of tendon for example) behave on "melting" very much as do fibres of elastoidin. The thermoelastic behaviour was studied by Wöhlisch (see 1932 for complete references), but little attempt was made to interpret the behaviour observed in terms of molecular structure until the work of Meyer & Ferri (1936) which demonstrated the parallel between the thermoelastic properties of thermally retracted tendon (treated with formaldehyde before melting) and those of vulcanized rubber.

(iii) *Elastin.*

Fibres such as those composing the *ligamentum nuchae* or occurring in connective tissue as elastic fibres offer in their elastic properties a close parallel to vulcanized rubber, elastoidin II, formaldehyde-treated tendon after "melting", etc. (Meyer & Ferri, 1936). At ordinary temperatures the fibres show high reversible extensibility, and from their thermoelastic behaviour it is clear that the force tending to restore the stretched fibre to its original length has its origin in thermal agitation, since it increases with rising temperature. If the fibres are stretched by about 65 %, the elastic properties change to those of a normal elastic solid; the restoring force under these conditions is due to the increase in potential energy of atoms or groups of atoms displaced by stretching from positions of minimal potential energy, whereas at smaller initial elongations (10–50 %) and at temperatures between 40

and 60° C., the internal energy remains practically unchanged on stretching. At an elongation of 65%, the elastic force, therefore, diminishes with rising temperature as in the case of a normal elastic solid.

It is interesting to note that elastin fibres contract on heating under zero load; that is, the resting length of the fibres diminishes with rising temperature. In the case of rubber and muscle the negative coefficient of linear expansion appears only when a certain elongation has been reached; unstretched or at very slight elongations they behave as normal elastic solids. Meyer & Ferri regard this behaviour of the *ligamentum nuchae* as due to the histological complexity of its structure. The thermoelastic behaviour corresponds to that of a system in which a contracting rubber band compresses a steel spring; on warming this system the elastic force of the rubber band increases while the resistance of the spring to compression is reduced; the overall length, therefore, diminishes. Microscopic examination reveals that collagen fibres are disposed in the substance of the ligament at right angles to the course of the elastin fibres. Thus the contraction of the elastin fibres will be opposed by the resistance of the collagen fibres. This resistance will diminish on warming and, as a result, the resting length of the system will also diminish. The behaviour of the ligament might also be produced by a similar arrangement on the molecular scale, that is, by transversely orientated chains impeding the shortening of the longitudinally disposed, flexible chains.

### (*b*) *Laminar structures*

Of the laminar structures built up from polypeptides, few have been investigated in detail as yet. An interesting example of a complex laminar protein structure arising extracellularly is provided by the egg-cases of selachians, recently studied by Fauré-Fremiet and his collaborators (see Fauré-Fremiet & Baudouy, 1938). It has been shown that the egg-capsules of three different elasmobranchs (*Scylliorhinus canicula, Raia batis* and *R. undulata*) consist of a keratin (ovokeratin) which, from the point of view of its sulphur content, may be regarded as a "soft" keratin, and which is formed from the secretion (prokeratin) of the nidamental glands (Filhol & Garrault, 1938).[1] The capsules are formed of three to four layers which may, according to the species, appear as sets of apparently homogeneous laminae, as an alveolar network, or as bundles of fibres separated by a system of canals. In the first case, which corresponds to the condition of all the layers in *Scylliorhinus*, the keratin chains in successive layers are orientated either transversely or longitudinally with respect to the axis of the capsule. This is clear from the optical and mechanical properties of the layers. Filhol & Garrault have related the different types of keratin structure formed to definite regions of the nidamental glands.

The basal membrane lying below the epidermis in selachian embryos, which has been studied by Garrault (1936), provides an example of a laminar structure arising

[1] In a private communication Dr W. T. Astbury informs me that X-ray photographs of fibres from the egg-cases show that these fibres belong to the collagen group and should not be referred to as keratins.

as a single sheet of microscopic interlacing fibrils forming a trellis in which the fibrils are arranged at right angles to each other. From their staining reactions and resistance to trypsin these fibrils appear to be collagen. The basement membrane seems to be intimately concerned in the orientation (in one plane at least) of the elastoidin fibres. Such a laminated structure may have considerable mechanical strength; if stretched in any direction it will not readily split. A single lamina would show high tensile strength in one direction—that in which the chains are orientated—but would split readily if stretched in the direction at right angles to the axis of the chains. A structure in which the chains in successive laminae run at right angles to each other will exhibit high tensile strength in two dimensions.

### (2) POLYSACCHARIDES

#### (a) *Fibrous structures*

The structure of chitin has been made clear from the chemical investigations of Bergmann *et al.* (1931*a*, *b*), Zechmeister *et al.* (1932, 1933) and Meyer & Wehrli (1937); from studies of the optical properties of animal chitin by Möhring (1926), who showed that chitin fibrils possess positive structural birefringence (with respect to the long axis), and negative intrinsic birefringence; and through X-ray studies, by Gonell (1926), by Meyer & Pankow (1935) on apodemes from *Palinurus vulgaris*, by van Iterson *et al.* (1936) on sporangiophores of *Phycomyces* sp., and simultaneously by Heyn (1936). It is clear from the results of these various lines of attack that animal and plant chitins are essentially similar and that, as was suggested by Meyer & Mark (1928*c*), chitin closely resembles cellulose in structure (Fig. 4(*b*)). Both consist of long primary valence chains of glucose residues. Chitin may be derived from cellulose by supposing a molecule of acetamide to be condensed with every glucose residue of the cellulose chain. The differences in three-dimensional structure between chitin and cellulose are due, as Heyn has shown, to the introduction of the side chain $CH_3.CO.NH$—.

Chitin appears in animal structures mainly in the form of fibrillar lamellae; isolated fibres are seldom encountered. One of the few examples of the latter are the fibres secreted by the ectodermal cells of the peduncle in certain barnacles (see Garrault, 1934, on *Pollicipes cornucopiae*).

#### (b) *Laminar structures*

To discuss in detail the structure of chitinous membranes would necessitate a description of microscopic particularities which space precludes. Early X-ray work on structure was done by Gonell (1926) on the elytra of *Goliathus giganteus*. In some cases, as Clark & Smith (1936) have shown, the long axes of the crystallites lie in the plane of the lamina, but the micells are otherwise distributed at random. In other cases (as in the elytra of *Goliathus*) the laminae are composed of fibrils all orientated in the same direction; crystallites of successive laminae make a constant angle with each other.

Cellulose occurs in the animal kingdom, forming laminated membranes in the test of tunicates. Mark & von Susich (1929) showed from X-ray studies that two sets of crystallites are present, lying with their long axes in the plane of the test and orientated at right angles to each other. From an examination of sections of the test in polarized light it is clear that, in some species at least, the laminae consist of crystallites all orientated in the same direction, but that the axes of orientation are at right angles to each other in successive laminae. In certain cases the two orientations of the crystallites are respectively parallel and at right angles to the long axis of the branchial basket (Herzog, 1932).

## VI. SUPRACELLULAR STRUCTURES

### (1) Fibrous structures

#### (a) *Hair, horn and feathers*

We shall begin this discussion of macrofibres with a survey of work by Speakman, Astbury and their collaborators on hair, horn and other keratinous structures. A hair provides a typical example of the structure intended by the expression "supracellular fibre". It consists essentially of concentric sheets of keratinized spindle-shaped cells with their long axes parallel to the hair axis. The whole is coated on the outside with a layer of scale cells—flattened, keratinized, epithelial cells. References to early studies of the optical properties of hair will be found in Schmidt (1924*a*) and to more recent work in Schmidt (1932*c*, 1934). A hair shows positive uniaxial birefringence, and from longitudinal sections it is clear that the more distal part of the hair is more birefringent than the proximal portion; this observation may be correlated with increasing keratinization, dehydration and orientation of the more distal keratin fibrils. All swelling agents lead to a fall in birefringence (Pochettino, 1913), while stretching causes an increase. Moderate warming produces a diminution in diameter and a rise in birefringence: heating very strongly induces thermal retraction, which Schmidt compares to that of collagen and elastoidin fibres.

The work of Astbury and his collaborators on the keratins has led to a picture of protein structure which appears to be applicable to many kinds of fibre. Astbury & Street (1931) showed that the rudimentary diffraction pattern given by normal hair is converted on stretching into a pattern closely resembling that of silk. Meyer & Mark (1928*b*) showed that the X-ray diffraction pattern of silk fibroin might be supposed to arise from a structure consisting of long, straight polypeptide chains (for the most part consisting of alternating glycyl and alanyl residues) associated to form elongated micells and arranged with their long axes parallel to the fibre axis. Astbury & Street (1931) and Astbury & Woods (1933) suggested, therefore, that the polypeptide chains are straight in the stretched hair ($\beta$-keratin)—hence the resemblance of the X-ray pattern to that of silk fibroin—but folded in some way in the normal unstretched hair ($\alpha$-keratin). Astbury & Woods (1933) offered reasons for supposing that the main polypeptide "backbones" are united by side chains with an average length of 9·8 A to form two-dimensional "grids" (see Fig. 5). In

β-keratin such grids are piled together (spaced at 4·65 A) to form tabloid micells (Astbury & Sisson, 1935) much thicker in the direction of the backbone spacing (4·65 A) than in the direction of the side chain spacing (9·8 A). The proof that these spacings are at right angles to each other was obtained by squashing horn in steam (Astbury & Sisson, 1935) and taking X-ray photographs with the beam parallel to the fibre axis, parallel to the direction of compression and at right angles to this direction. This molecular interpretation of the changes in the X-ray diffraction patterns of hair on stretching has been extended to similar changes observed in myosin films and, later, in dried muscles stretched in cold water (Astbury & Dickinson, 1935 *a*, *b*; 1936).

The normal folded state of the keratin grid is supposed by Astbury to be due mainly to the presence of buckling linkages, both cross-linkages and linkages in the length of the chains. Among these linkages the cystine side-chains have been shown to be of great importance. On heating in steam, certain linkages are hydrolysed, and

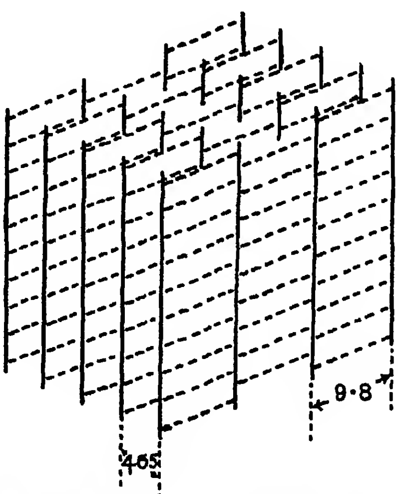



Fig. 5. The structure of β-keratin: stretched keratin grids consisting of polypeptide backbones held together by side-chains are stacked 4·65 A apart to form minute elongated tabloid micells. The structure is to be continued in three dimensions. The spacings are in angstroms ($10^{-8}$ cm.).

the grids may be straightened out. (This hydrolysis also takes place, but of course more slowly, at lower temperatures.) Deforming linkages are stretched and, lubricated by water molecules, the chains untwist. When the deforming force is removed the grid returns to its contracted and crumpled form as a result of the increased potential energy of the distorted parts of the system in the stretched condition. It is to be noted that this view of the mechanism of elastic contraction in hair is fundamentally different from the interpretation of high reversible extensibility which has been advanced by Meyer and his collaborators in the case of other high polymers.

A stretched hair, heated in steam and allowed to contract unloaded, reaches a length shorter than its original length (supercontraction: Astbury & Woods, 1933). Since muscle and myosin fibres give an X-ray diagram resembling that of α-keratin, it has been suggested (Astbury & Dickinson, 1935*a*) that the active contraction

of muscle may be compared with the supercontraction of keratin, rather than with the change from $\beta$- to $\alpha$-keratin.

The X-ray diffraction pattern of feather keratin (Astbury & Marwick, 1932; Marwick, 1933) indicates that the keratin chains are almost fully stretched in this case—the spacing along the fibre axis is 3·1 A as compared with 3·3 A in $\beta$-keratin and 3·5 A in silk fibroin. In addition the pattern shows periodicities of twice, four and eight times this unit along the fibre axis. Astbury & Marwick interpret these larger spacings as an architectural relic of a previous globular protein structure from which the keratin chains have been derived by a process of "denaturation". Of particular biological interest is the resemblance between the pattern of feather keratin and that of reptilian keratin (tortoise-shell, Marwick, 1933). The separation of Sauropsida from Theropsida has thus its molecular aspect. From this point of view it would be interesting to examine the structure of reptilian and mammalian scales. Astbury has shown that the keratin chains in finger nails ($\alpha$-keratin) run in the plane of the nail, transverse to the axis of the digit—hence transverse splitting.

Astbury & Woods (1933) consider that the keratin of the hair is present in three phases: (*a*) intracellular, (*b*) keratinized cell walls, and (*c*) intercellular keratin. From the point of view of its elastic properties the hair behaves as a continuous network of molecules. There is, according to these authors, "no sharp line of demarcation between the phases. The phases differ in their resistance to chemical attack and in the regularity of their 'crystalline' structure." But, "to a first approximation we may consider the generalized load/extension of the hair as that of a single-phase transformation comparable with that of rubber". (See also the discussion of this point in the introduction to the paper by Wood, 1938.) The importance of this conclusion from a biological point of view is that the hair cells are not sharply separated one from another. Woods (1938) has shown that the keratin chains in individual spindle-shaped hair cells are orientated parallel to the long axis of the cell, and it is to be supposed that the keratin chains of individual cells are linked together from cell to cell so that, in a sense, the hair forms a single gigantic molecule, no doubt with free ends of polypeptide chains here and there. From the molecular point of view the cell disappears as a discrete entity in the finished hair. It is for this reason that the concept of supracellular organization has been introduced, reviving in a sense Heidenhain's concept of histo-systems above the cell level.

### (*b*) *Muscle*

The elastic behaviour of the resting muscle as a whole leads us to regard the system which is responsible for the transmission of tension from one end to the other as a continuous molecular network with free chains in the meshes of the net (Meyer & Picken, 1937). This means that, in spite of its histological complexity, the muscle is a continuous molecular system. We must suppose (1) that the myofibrils are in molecular connexion with the collagen fibres in which the muscle fibres terminate, and (2) that the muscle fibres are linked one with another by molecular fibrils of collagen. The histological grounds for assuming this continuity of structure are discussed by Schmidt (1936*d*). In the case of insect muscles it is known that the

muscle fibres continue into the insertion of the muscle as protein fibrils (tonofibrils). In this case, therefore, we may assume continuity between tonofibrils and chitin chains.

### (c) *Enamel prisms*

Studies of the denticles from uncut elephant molars have been made by Schmidt (1934), and have shown that each enamel prism is negatively birefringent and uniaxial, but is not to be regarded as an individual crystal. Prisms from the enamel of immature mammalian teeth exhibit positive structural birefringence as well as negative intrinsic birefringence, and it is clear from the study of sections that both structural and intrinsic birefringence are uniaxial. During hardening of the enamel the structural birefringence and the ability to take up liquids both diminish. The

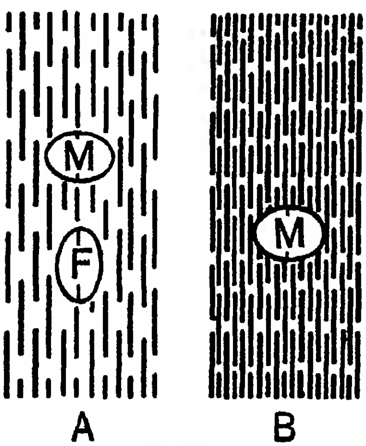



Fig. 6.

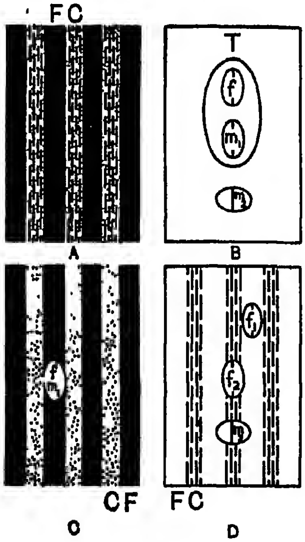



Fig. 7.

Fig. 6. The structure of enamel (from Schmidt, 1934). A. Young enamel; strokes represent hydroxy-apatite micells. The structure shows positive structural birefringence ($F$) and negative intrinsic birefringence ($M$) (sign with respect to the long axis). B. Mature enamel; this shows intrinsic birefringence only, since the spaces are occluded.

Fig. 7. The structure of bone (from Schmidt, 1934). A. Normal bone; parallel collagen fibres, $F$, are surrounded by a matrix, $C$, consisting of hydroxyapatite micells (strokes) and organic material (dots). The optical behaviour is shown in B, where the ellipse, $T$, represents the total birefringence of the collagen fibres composed of positive structural ($f$) and positive intrinsic ($m_1$) birefringence; $m_2$ is the negative intrinsic birefringence of the mineral constituents. C. Decalcified bone; the collagen fibres, $F$, showing positive structural ($f$) and intrinsic ($m_1$) birefringence are separated by canals containing the organic constituents of the cementing material. D. Bone freed from organic constituents. The material shows positive structural birefringence, $f_1$, resulting from the fine tubules once occupied by collagen, negative intrinsic birefringence, $m_2$, due to the hydroxyapatite micells (strokes) and positive structural birefringence, $f_2$, from the spaces between the inorganic micells once occupied by organic cement.

changes appear to be due to the reduction in size of the intermicellar spaces, either by the growth of the micells or by the formation of new crystallites between the old (see Fig. 6). The negative intrinsic birefringence appears to be due to the hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) micells which constitute 90% of the enamel.

X-ray analysis (Möller & Trömel, 1933) supports the conclusion that the hydroxyapatite micells are arranged parallel to the axis of the prism.

Young decalcified prisms from elephant molars show faint positive birefringence (in water and in balsam) which may be due to the organic constituent. Schmidt suggests that the regular orientation of the hydroxyapatite micells may take place, as in the case of bone, by orientated adsorption of organic matter on the processes (Tomes's processes) which develop from the odontoblasts. The strong dichroism of young prisms stained with congo red or impregnated with silver confirms the picture of their micellar organization outlined here.

Schmidt (1938*a*) has also investigated the development of the enamel-like dentine in cyprinoids.

### (2) Laminar structures

#### (*a*) *Bone*

The birefringence of sections of normal bone is largely due to the presence of positive, uniaxial collagen fibres (von Ebner, 1894). In the simplest case, the laminae of a single Haversian system consist of collagen fibres running parallel to and at right angles to the axis of the canal in successive laminae (see Schmidt, 1934). In section (viewed between crossed nicols) the laminae will be alternately dark (fibrils cut at right angles to the optical axis) or bright. Where the fibres run parallel to the plane of vibration of polarizer or analyser, extinction is complete and, therefore, the Haversian system as a whole shows a negative spherite cross. In general, however, the structure is more complex, the fibrils are usually disposed in steeper and less steep spirals (in successive laminae), and no lamina, therefore, is completely isotropic when seen in section. Carefully decalcified sections show optical properties resembling those of normal bone, and hence it is concluded that the birefringence of the latter is due for the most part to the collagen fibres present (Fig. 7 C). Decalcified material shows many phenomena found in collagen fibres (reversal in sign of the birefringence on treatment with phenol, clove oil, salicyl-aldehyde, etc., von Ebner, 1894). Sections will also show dichroism if impregnated with gold, etc. (Schmidt, 1931).

If sections of bone are carefully freed from organic matter (in particular from collagen), they are found to show structural and intrinsic birefringence (Fig. 7). The former is due to the presence of minute tubes which once contained the collagen fibrils; the latter to the micells of hydroxyapatite. This is evident from the optical behaviour in liquids of various refractive indices. In methylene iodide ($n_D = 1{\cdot}74$) the birefringence is positive, as it is in alcohol ($n_D = 1{\cdot}33$), but in xylol ($n_D = 1{\cdot}49$) the birefringence is negative. It is found that both structural and intrinsic birefringence are uniaxial, and that the optical axis is parallel to that of the collagen fibres. Careful study of sections embedded in a medium of refractive index equal to that of the inorganic matrix has shown that, in addition to the relatively gross structural birefringence, the matrix possesses positive structural birefringence arising from the minute regular spaces between the inorganic micells. It may be concluded that the

micells of hydroxyapatite, which are negatively birefringent, are anisodiametric and are arranged with their long axes parallel to the optical axis of the collagen fibrils, and that intermicellar spaces of the same shape and orientation occur between the inorganic micells (Fig. 7A, B).

Schmidt (1934) suggests that the simplest explanation of the observed orientation of the inorganic crystallites is to suppose that the first-formed collagen fibrils adsorb the crystalline precipitate of inorganic matter in an orientated manner, and he shows that collagen fibres are able to orientate particles precipitated in their presence (see also Kohlschütter, 1929). Decalcified sections moistened with xylol and gently warmed with a fragment of paraffin wax become negatively birefringent if the wax particles formed on cooling are not too large; the change is produced by the orientated adsorption of the wax crystallites. Orientated adsorption of wax or congo red may also be demonstrated in sections freed from organic matter.

## VII. DISCUSSION

It is clear from the material which has been surveyed that a remarkably close parallel exists between the structure and behaviour of biological materials and of high polymer substances in general. Many of the peculiar mechanical properties of "living substance" take their place beside those of synthetic inorganic and organic substances which owe their distinctive properties not to the chemical nature of their component atoms but to the fact that their molecules are of a certain shape. The elastic behaviour of muscle or of the cell surface, intermediate as it is in certain respects between that of a solid and that of a liquid, receives a simple explanation in terms of the properties of the long flexible molecules of certain types of high polymers; such molecules are in a sense "liquid" in two dimensions—able to slide over their neighbours as freely as the molecules in a liquid—but "solid" in a third dimension, since they are composed of atoms or groups of atoms united by primary valences to form a chain. We find then physicochemical evidence for the view—by no means strange to the biologist—that the properties of living matter depend on structural relationships of a complexity unlike anything which we meet in the dead world and which we have only recently succeeded in imitating in inorganic models.

The study of high polymers affords the biologist insight into the mechanical properties of the materials with which he is concerned and some measure of understanding of their structural potentialities—their tendency to form crystallites, fibrils, fibres and laminae. The micellar constitution of a great variety of biological structures has now been confirmed by evidence from X-ray analysis, from elastic and thermoelastic properties, as well as from birefringence and imbibition data. In the case of cell components which are as yet only accessible to optical methods of structural analysis, we have evidence of micellar fibrous structure from their behaviour during the life of the cell—that is, from phenomena which are usually regarded as essentially biological, but for which we find parallels in the behaviour of synthetic high polymers. We may recall the longitudinal splitting of chromosomes and flagella, or the change in any contractile fibre in which the passage of orientated

chain molecules from the stretched condition to more random configurations leads to a change in shape of the fibril, that is, to contraction.

If then we can describe the behaviour of a contractile fibril, for example, in terms of its molecular structure, it is evident that, at the level of molecular organization, function and structure are inseparable. The contraction of a muscle fibre is ultimately as much a morphological as a physiological problem: we are faced with the analysis of a complex of physicochemical conditions, change in which leads to changes in the molecular morphology of the myosin fibrils. In the same way certain aspects of the behaviour of the chromosomes are closely related to their structure; the changes from resting nucleus to the appearance of the earliest prophase threads can be considered as changes in the molecular organization of the chromatin, as phenomena of swelling and dehydration (see Schmidt, 1937*b*). It is apparent that as yet certain aspects only of the activities of the cell appear in a morphological guise, namely those which are associated with movement or more generally with changes in shape.

Although the ordered and in some respects microcrystalline character of many biological structures has been emphasized in these pages, this does not imply that the organism as a whole is to be regarded as a crystal-like structure—a view which has been worked out in some detail by Przibram (1926). Because its parts are microcrystalline, the wholeness of the organism is not necessarily the wholeness of a crystal (if there is any such thing). Nevertheless, it seems clear that the form of cell components may be decided by factors similar to those which fix the form of true crystals, and that the shape of the cell components is determined by the relative velocities of growth in three dimensions of ordered aggregates. The factors responsible for the formation of a flagellum are perhaps related to those which determine that a given crystal shall grow as a needle rather than equally in all three dimensions. Schmidt (1937*a*), discussing the formation of filopodia in the foraminiferan *Miliola*, rejects the view that mechanical stretching of the chain molecules leads to growth of the filopodium. He suggests rather that the pseudopodium grows in length as a result of some kind of "micellar crystallization"—like a thread of fibrin. He supposes that the protoplasm contains elongated micells which are bundled together in the axis of the pseudopodium to form a solid core. The superficial protoplasm remains liquid, and streaming brings fresh micells to be added to the distal end of the pseudopodium.

Garrault (1937), in a discussion of the development of fibrils in the basement membrane of the epidermis in selachians, remarks that the fibrils may be regarded as collections of crystallites approximately parallel one to another. In the last analysis the orientation of a fibril is equivalent to the orientation of a molecular structure, but it is clear that the orientation depends on the morphological properties of the embryo considered as a whole. Here, just as in the case of pseudopodium formation, there remains the fact that the fibrous structure, even if it grows as a crystal, develops in one particular place and with a particular orientation with respect to the organism as a whole. It is precisely the question of how such co-ordination takes place which is the central problem of biological organization.

Supposing that the growth of the cell components is governed by differential rates of micellar crystallization, it is evident that the changes in shape of cells or parts of cells may also be explicable on this basis. In the case of one type of cell component, it is possible to compare an analysis in terms of the field concept of the change in shape during development with a fine structure analysis of the same process. Gurwitsch (1927) seeks to describe the conversion of a spherical sperm head into the hook-shape of the mature sperm (rodent). He does so by placing the sphere in a conical field with the centre of the field (towards which movements are directed) lying at the apex of the cone. The shape of the head changes by displacement of points on its surface along the lines of the field, the displacement being greatest along the axis of the cone (that is, the field has a certain "anisotropy"). The hook shape may be obtained by supposing the field centre to shift through an arc, with the result that the direction of maximum displacement changes.

Schmidt's analysis of similar morphological changes presents striking contrasts to such a purely geometrical approach. Studies of the optical properties of the sperm head of *Sepia* showed clearly that the chromatic portion possessed an orderly micellar structure; the individual chromatin micells (showing negative birefringence) are orientated parallel to the axis of the sperm head, which is very slightly curved at the anterior end. In the presence of acid the sperm head swells laterally and shortens, eventually becoming spherical; during these changes the birefringence is greatly reduced or disappears. This behaviour is obviously reconcilable with fibre structure. Schmidt (1928*c*) and Pattri (1932) showed, as a result of the examination of sperm from about eighty different animals from all the main phyla, that the birefringence is always negative with respect to the length and strongest in thread-shaped heads. Furthermore, extinction between crossed nicols always occurs in a plane parallel to the axis of the sperm head, whether this be straight, curved or spirally twisted; this means that the shape of the sperm head is always intimately related to the micellar organization of the chromatin. Schmidt found in sperm from *Triton cristatus* that the pointed anterior end alone was negatively birefringent, the broader, more swollen, posterior end being positively birefringent (positive structural birefringence). In 0·9% sodium chloride the heads swelled and became uniformly positively birefringent; on dehydrating with alcohol the birefringence became uniformly negative. These observations indicate that the state of micellar organization may vary from one part of the sperm head to another, and that shape and structure vary together.

In the course of spermatogenesis in *Liogryllus*, Pattri (1932) found that the birefringence first appeared when the head became elongated, and from the brightness of the outline of the head Pattri supposed that "dehydration" proceeds from the surface inwards. It is to be concluded that the change in shape of the sperm head is a consequence of the chromatin micells assuming an orientated arrangement during the process of dehydration. Indeed, as Goldschmidt (1917) showed, the immature sperm may be induced to assume a form closely resembling that of the mature sperm by placing the testicular material in a medium of high osmotic pressure. We cannot say as yet what determines the formation of a curved or spiral

micellar aggregate, or why parts of the head are less dehydrated than others, but it is perhaps a little premature to seek refuge in a purely geometrical description of the process of spermatogenesis.

The case of the sperm head is at the moment the only one in which the development of a characteristic shape of cell component has been correlated with changes in micellar organization. There are, however, many examples in which the development of fibrous structures leads to changes in shape of the whole organism. In spermatogenesis, the growth of the axial filament carrying out the protoplasm to form the tail may be compared with the growth of a heliozoan pseudopodium (see Goldschmidt, 1917; Herrmann, 1890; Spitschakoff, 1909). But one-dimensional fibre growth may be important in determining the shape of more complex forms; in the development of the echinopluteus, it is the growth of the skeletal rudiment which pushes out the surface into arms.

The demonstration by Meyer (1867) of the correspondence between the microscopic structure of bone and theoretical diagrams of the tension and compression lines in a hypothetical structure subjected to like forces receives a simple explanation when we realize that the collagen chains will, of necessity, be orientated by the mechanical forces acting on the system. Once their orientation is fixed, the deposition of salts follows the orientation of the collagen chains. It is extremely probable that the orientation of other primary valence chains (other polypeptides, chitin and cellulose, etc.) is due to forces acting during the deposition of the substance in question. Thus the apodemes of Crustacea always show a high degree of orientation, the long axis of the chitin micells lying parallel to the direction of the tension set up by the contracting muscles; the integument shows no such orientation (Clark & Smith, 1936), the crystallites lie with their long axes in the plane of the surface, but these axes are not parallel to one another. Again, the chitin fibrils in successive lamellae of many lamellar membranes in insects cross at 90° or 60°; no one has as yet investigated the relation between the direction of the compression and extension forces acting on particular membranes and the orientation of the chitin chains. If, as seems highly probable, such orientation is due to forces acting while the chitin is being deposited, it is clear that not only the immediate pull of the muscles on the soft chitin will be important in determining orientation, but also the hydrostatic pressure of the body-fluid acting over the surface of hollow cylinders (bodies and limbs of worms and arthropods). The problem is essentially the same, mechanically, as that presented by the orientation of cellulose and chitin chains in plant cell walls (*Valonia*, Astbury *et al.* 1932; *Phycomyces*, Heyn, 1936; Castle, 1937), where it is certain that turgor plays an important part in the development of orientation (see van Iterson, 1936, and the discussion in Preston & Astbury, 1937).

In many cases, however, it is clear that orientation takes place without the intervention of any such external mechanical forces. The problem may be expressed in the form of a question: how far are the changes in shape of cells, cell components or extracellular structures the result of external forces leading to the orientation of micells, and how far to "crystal-like" growth in particular directions determined by the spatially differentiated regulatory system which is responsible for the

organization of the whole? The question is the same as that asked by Schmidt in considering the formation of a pseudopodium. From our knowledge of non-living systems, we should expect both processes to play their part in morphogenesis.

To those who find only destructive atomism in such attempts to analyse developmental processes, it may be pointed out that the physics and chemistry of high polymers, to which constant reference has been made, is to some extent the private physics and chemistry of the biologist. Rubber-like high polymers do not occur in the dead world; their synthesis from inorganic materials has only recently been accomplished. Our knowledge has come from the study of such things as cellulose, rubber, chitin, silk, hair and muscle. The relations between structure and shape which we have discussed must be regarded as part of that system of organizing relations which forms the main objective of the biologist's approach to living things.

## VIII. SUMMARY

1. Present-day interest in the study of fine structure may be traced back to the work of the botanist, Carl Nägeli, to whom the conception of the micell is due.

2. The continuation of Nägeli's optical studies of biological structures, the development of X-ray analysis, and the investigation of the properties of long chain high polymers, have led to the extension and modification of Nägeli's ideas.

3. Two main types of molecular aggregate are of particular importance in biological systems—linear (or fibrous) and laminar structures. These may be classified as subcellular, extracellular and supracellular.

4. The subcellular structures which have been examined include: protein fibres from the sap of virus-infected plants; chromosomes; asters and spindles; and contractile fibrils (muscle fibrils, myonemes, pseudopodia and cilia). These all appear to be composed of chain molecules arranged approximately parallel to the long axis of the structure in question.

Laminar subcellular structures include the surface membranes of animal cells (nerve fibres, echinoderm eggs, red blood corpuscles) and the outer portion of the rods and cones of vertebrate retinae. These are composed apparently of alternating layers of protein and lipoid molecules.

5. Extracellular fibrous structures are represented by elastoidin, collagen, elastin and chitin; laminar structures by the keratinous egg cases of selachians, the cellulose test of tunicates and chitinous integuments in general. The relation of these to other natural and synthetic high polymers is discussed.

6. The category of supracellular structures, that is, of macroscopic fibrous or laminar structures of multicellular origin includes (*a*) hair and muscles, and (*b*) enamel and bone.

7. It is clear that a close parallel in structure and behaviour exists between biological materials and high polymer substances. The morphological implications of this parallel are discussed and the importance of molecular morphology for the biologist is considered.

I am much indebted to all those who have read and criticized this review. My special thanks are due to Dr W. T. Astbury of Leeds, Professor Kurt H. Meyer of Geneva, and Professor W. J. Schmidt of Giessen for their invaluable counsel.

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# CYANIDE INHIBITION AS A MEANS OF ELUCIDATING THE MECHANISMS OF CELLULAR RESPIRATION

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(*Received* 23 *June* 1939)

CONTENTS



## I. INTRODUCTION

It has been known for some time that the physiological properties of hydrocyanic acid and its salts are marked by a specific effect on respiration. Gaethgens (1868), Claude Bernard (1883) and Geppert (1889) called attention to the inhibitory effect of cyanide on the respiration of higher animals. In subsequent years it has been shown that cyanide exerts a specific inhibition upon certain enzymatic oxidative processes.

A. P. Mathews and his co-workers (Mathews & Walker, 1909) described the inhibitory effect of cyanide on the iron catalysis of cysteine oxidations and ascribed the phenomenon to the formation of a complex Fe—CN ion. Soon thereafter, Warburg (1914), in a series of extensive researches, pointed out that many cellular oxidations were catalysed by an iron-containing enzyme which he termed the "Atmungsferment". One of the chief diagnostic features of this enzyme was its extreme sensitivity to inactivation by cyanide. Since then the work of Keilin (1929, 1930, 1932), Dixon (1929) and others has resulted in the elucidation of a respiratory system (the "Warburg-Keilin" system) which involves a number of interrelated oxidative enzymes and carriers. The general features of this system may be represented as follows:

$$O_2 \rightarrow \text{Oxidase—}\underset{(a-b-c)}{\text{Cytochrome complex}}\text{—Dehydrogenase} \leftarrow \text{Substrate metabolite.}$$

According to this scheme,[1] the substrate (e.g. a carbohydrate) is rendered liable to oxidation by the action of a dehydrogenase enzyme specific to it. Presumably this action is a kind of "loosening" of the H atoms. The cytochrome complex "accepts" these H atoms and is thereby reduced. The reduced cytochrome itself reduces the oxidase from a "ferric" to "ferrous" state. (This enzyme is Warburg's original *Atmungsferment* and is variously known as cytochrome oxidase, indophenol oxidase or pheohemin.) The reduced cytochrome complex is thereby reoxidized and prepared for further carrying of the oxidation-reduction process. Finally, the reduced oxidase is reoxidized by molecular oxygen. The net result of this process is the oxidation of the metabolite substrate and the reduction of molecular oxygen. Thus, the metabolite is consumed, oxygen is absorbed and carbon dioxide and water are produced. The rate of respiration effected by this system may be measured by the rate of oxygen consumption.

The effect of cyanide was shown to be localized at the oxidase by Fleisch (1924), Szent-Gyorgyi (1924) and Keilin (1929). It was observed that the dehydrogenase activity was not affected by cyanide if the oxidase were replaced by a dye capable of reduction by the metabolite and its dehydrogenase, and of oxidation by molecular oxygen (e.g. methylene blue). This has recently been confirmed by Leloir & Dixon (1937). The cytochrome carrier persists in its reduced form if cyanide is present, but is not itself attacked by the poison (Keilin, 1929). It has therefore been established that the oxidase alone is inactivated by cyanide. Thus, the presence of cyanide prevents the reoxidation of reduced cytochrome and oxygen consumption is thereby blocked. Consequently, cyanide will inhibit the respiration that is mediated through any system which utilizes molecular oxygen by way of the cytochrome oxidase.[2] Hence, if cyanide (or a similar poison) is present in a concentration sufficient for the complete poisoning of the oxidase, any remaining oxygen consumption must be mediated by respiratory systems which do not involve the oxidase. This "residual" or "cyanide-stable" respiration must therefore be mediated through enzyme systems which are capable of reducing molecular $O_2$ through some independent means.

Dyes such as methylene blue (which are artificially introduced) may perform this function. The most important of the naturally occurring substances of this sort is the so-called "yellow enzyme" or flavoprotein of Warburg & Christian (1932). This is a protein combined with a riboflavin prosthetic group. It is directly oxidizable by molecular $O_2$ and itself oxidizes a "coenzyme" (a tri-phospho-pyridine nucleotide) which in turn oxidizes the metabolite substrate (through its dehydrogenase). The yellow enzyme is not affected by cyanide and may act as a mediator of cyanide-stable cellular respiration.

[1] This scheme does not include certain carriers which operate within the system. Thus Szent-Gyorgyi (1935) has shown that fumaric, succinic and oxalacetic acids may form an intermediary chain in the oxidation and reduction process that goes on between the cytochrome complex and the dehydrogenase. Other reversible systems may perform a like function; but these as well as the detailed relations between cytochromes *a*, *b*, and *c*, are omitted for the sake of simplicity. They are not essential to the consideration of the present problem.

[2] Carbon monoxide and nitrous oxide have a similar effect on the oxidase.

There is some *in vitro* evidence that the oxidation of metabolites may be carried out by a series oxido-reduction of both systems. Thus, Theorell (1937) shows that the yellow enzyme's coenzyme may sometimes be coupled with the cytochrome oxidase and oxidized by the latter. Dewan & Green (1938) suggest that a "coenzyme factor" catalyses the reduction of the cytochrome complex by the coenzyme of the yellow enzyme. Several such complex series have been evolved by means of various *in vitro* experiments. It has often been suggested that the various oxidative enzymes and carrier systems are linked in a series according to their respective redox potentials (as measured *in vitro*). For a recent summary of this point of view see Barron (1939).

However, the very fact that the respiration of most cells is only *partially* inhibited by cyanide permits of the use of the concept of a "cyanide-sensitive system" and a "cyanide-stable system". That is, regardless of the inner complexities and the precise course of the respiratory processes, this fact indicates that the total respiration may be accounted for by two enzyme series—the one blocked by cyanide, and the other unaffected by this poison. However complicated and interrelated these two series may be, they are clearly distinguished by their means of reducing molecular oxygen. The "cyanide-sensitive system" accomplishes this through the cyanide-sensitive cytochrome oxidase, while the "cyanide-stable system" does so through an enzyme which is not affected by cyanide.

The extent of the cyanide inhibition of respiration would seem to indicate the relative activity of these two systems. In his early work, Warburg (1927) found that over 95% of the respiration of bakers' yeast (in a medium containing sugar) was inhibited by cyanide. This high sensitivity[1] led him to believe that the "Atmungsferment" accounted for almost all cellular respiration. This suggestion caused other workers, and Dixon & Elliott (1929) in particular, to test the hypothesis by determining the cyanide-sensitivity of various tissues. The fact that the *average* sensitivity of mammalian tissues was but 65%, led Dixon & Elliott strongly to dispute Warburg's conclusion. Since then the literature has contained a series of contradictory data and disputed conclusions on this subject. It is the purpose of this paper to analyse these data with the aim of providing a description of the relationships between the respiratory systems as they occur in the living cell. Further, it will be shown that cyanide inhibition may be used as a means of elucidating certain internal properties of the Warburg-Keilin respiratory system.

## II. THE RELATIVE RATES OF ACTIVITY OF THE CYANIDE-SENSITIVE AND CYANIDE-STABLE RESPIRATORY SYSTEMS

In the enunciation of his "Atmungsferment" theory, Warburg assumed that it was alone the mediator of all cellular respiration. Consequently he concluded that oxygen consumption should be completely inhibited by a low concentration of

[1] The term "cyanide sensitivity" is defined as the percentage of the total rate of respiration which is inhibited by cyanide, i.e.

$$\text{cyanide sensitivity} = \frac{(\text{original rate of respiration}) - (\text{rate in CN})}{(\text{original rate})} \times 100.$$

cyanide. This was found to be the case for the respiration of yeast in a sugar medium, but further research showed that there were many exceptions. As early as 1889 Linosier had found that cyanide sometimes did not inhibit respiration very extensively. A similar observation had been noted by Battelli & Stern (1907), who found that cyanide produced but a small percentage inhibition of the respiration of mammalian tissues. Warburg himself (1919) and Emerson (1927) demonstrated that respiration of the alga *Chlorella* was only slightly cyanide-sensitive. Lund (1918*a*, *b*) and Shoup & Boykin (1931) found the same to be true for the respiration of *Paramecium*.

Finally in 1929 Dixon & Elliott made many careful measurements of the respiration of mammalian tissue slices in phosphate-Ringer's solution and in this medium plus $M/300$ HCN. They found that inhibition varied from 25 % to 91 % for various samples of several tissues. Since the average inhibition was about 65 %, they concluded that: "The respiration of animal tissues is made up of two parts. One, accounting for about two-thirds of the total, is due to systems poisoned by cyanide; the other one-third is due to systems which are stable to cyanide. Warburg's 'respiratory enzyme' (Keilin's cytochrome-indophenol oxidase system) can therefore only account for at most about two-thirds of the total respiration of animal tissues." It was also shown that the respiration of "fed" baker's yeast (incubated in sugar, and respiration measured in the same medium) was inhibited about 95 % by $M/30$ KCN, while "starved" yeast (suspended in sugar-free buffer and aerated before use) was inhibited only 72 %.

The conclusions reached by Dixon & Elliott were sharply criticized by Warburg (1931) on the grounds that the use of phosphate, bicarbonate-free medium (since all carbon dioxide was absorbed by the KOH inset) injured the cells, and that the "Atmungsferment" of such "geschädigten Zellen" would consequently be in an abnormal state and not suffer complete cyanide inhibition. Warburg's student, Alt (1930), repeated the work of Dixon & Elliott, but with a bicarbonate buffer and an atmosphere containing carbon dioxide. (The two-vessel method was used.) Alt found that $N/100$ cyanide inhibited the respiration of kidney, liver and spleen, 96, 99, and 95 % respectively. The discrepancy between these results has been the subject of a long controversy, and has usually been ascribed to the different effect of phosphate and bicarbonate buffers.[1] It is, however, necessary to note here that another factor must be considered as well. Alt's respiration medium contained 2 % glucose, whereas that of Dixon & Elliott did not include a substrate. Unfortunately, no mention of this is made in the body of Alt's paper, and the presence of glucose is noted only in the appended protocols. As will be shown below, it is this which is at the bottom of the "discrepancies"; the substrate concentration is the critical factor.

[1] v. Heyningen (1935) has shown that rat tissue slices (liver and kidney) in a phosphate medium have a respiration rate that is 20 % lower than the rate in a bicarbonate medium. However, the rate in the presence of cyanide is the same in both cases. Thus it appears that phosphate inhibits the respiration by affecting the cyanide-sensitive system alone. This effect may in part contribute toward the low percentage inhibition found by Dixon & Elliott and other workers who have used phosphate media. This result is confirmed by the observations of Kisch (1933*a*).

Keilin (1932) suggested that cells that are saturated with carbohydrate metabolite appear to be more cyanide-sensitive than cells respiring in a substrate-free medium. This was known to be true for yeast (Warburg, 1927; Dixon & Elliott, 1929) and for *Chlorella* (Emerson, 1927 *a*, *b*). Keilin was unaware of the fact that Alt's medium contained glucose, and ascribes the above discrepancy to the buffer used.

The solution to these difficulties becomes apparent with a closer examination of the data of Alt and of Dixon & Elliott. The tables published by the latter show that slices of the same tissue show a wide distribution of respiration values in Ringer-phosphate alone. Thus, seven samples of sheep liver give rates varying from 360 to 1800 cu. mm. of oxygen per gram of fresh tissue per hour (i.e. a five-fold variation). On the other hand, the respiration of these same slices in the presence of $M/30$ KCN is far more constant, varying only from 61 to 91 (variation: 1·5 times) cu. mm. of oxygen per hour per gram wet weight. A similar effect is apparent in all their data, and it appears that all random variations in original rate of respiration are much larger than variations in the residual respiration in the presence of cyanide.

Consequently, since percentage inhibition $= \left(1{\cdot}0 - \frac{\text{Rate of respiration in CN}}{\text{Original rate}}\right) \times 100$,

slices that had a high original rate showed a high sensitivity (i.e. percentage inhibition) to potassium cyanide and *vice versa*. In order to test this relationship we may state that:

$$\text{Original rate of respiration} = \text{KCN-sensitive respiration} + \text{KCN-stable respiration}, \qquad (1)$$

(all of these values in terms of the *absolute* rates). If the absolute value of the cyanide-stable respiration remains constant while the original (KCN-free) rate varies, then the above equation may be written in the form

$$y = x + b, \qquad (2)$$

where $y$ = original (KCN-free) rate of respiration, $x$ = absolute value of the KCN-sensitive respiration, and $b$ = the absolute value of the KCN-stable respiration (which is constant). If this relationship holds, a plot of the original rate of respiration ($y$) against the absolute value of the KCN-sensitive respiration ($x$) should fall on a straight line with a 45° slope. Furthermore, the intercept of this line on the ordinate is equal to the constant $b$ and represents the true value of the cyanide-stable respiration.

The data of Dixon & Elliott are plotted in these terms in Fig. 1. It is apparent that the above relations hold quite well. They are equally descriptive of the variations between different tissues and the variations between different slices of the same tissue.

This relationship also serves to clarify the apparent discrepancy between the data of Dixon & Elliott and those of Alt. If the original rates of respiration are

compared, we find that the maximum $Q_{O_2}$[1] obtained by Dixon & Elliott is: for sheep kidney, −8·5; rat liver, −4·3; ox spleen, −1·5. The corresponding $Q_{O_2}$ obtained by Alt for rat tissues is: kidney, −19·4; liver, −15·1; spleen, −12·3. When the absolute values of the cyanide-sensitive respiration are calculated from

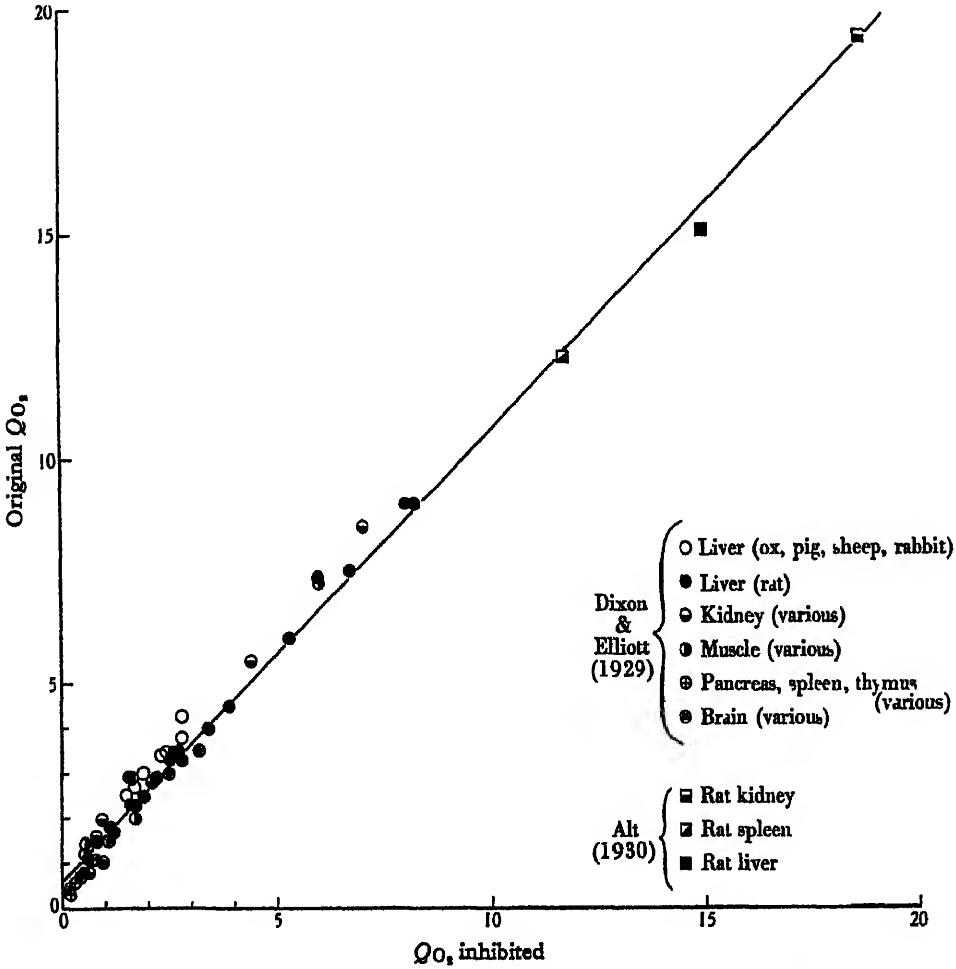


Fig. 1. Data adapted from Dixon & Elliott (1929) and Alt (1930). The medium used by Dixon & Elliott contained no substrate, while that used by Alt contained 0·2 % glucose. "$Q_{O_2}$ inhibited" equals the original $Q_{O_2}$ minus the $Q_O$ in cyanide.

the percentage inhibitions given in Alt's paper (see p. 172), the three upper points of Fig. 1 are obtained. It is clear that both sets of data follow the same relationship. The difference in *percentage* inhibition is merely a result of the difference in the original rate of respiration of the tissues. Thus, while the rates of respiration of

[1] The symbol "$Q_{O_2}$" is defined as the number of cubic millimetres of oxygen consumed per hour by an amount of tissue of 1 mg. dry weight. The data of Dixon & Elliott are converted to $Q_{O_2}$ by multiplying by 1/200, assuming an 80 % water content for wet tissue. The negative sign indicates that oxygen is absorbed.

Alt's tissues are much higher, the cyanide-stable respiration is equal to that found by Dixon & Elliott; i.e. points from both sets of data fall on the same straight line. The intercept of this line indicates that the absolute cyanide-stable respiration is represented by a $Q_{O_2}$ of about $-1 \cdot 0$.

Thus, percentage inhibition by cyanide depends on the original cyanide-free rate of respiration. The cyanide-stable respiration rate is relatively constant and low in value as compared with the original rate. Consequently, as the variable original rate increases, the percentage inhibition also increases and approaches 100 %.

Thus, the apparent discrepancy between the two sets of data is a consequence of the difference in original $Q_{O_2}$; and this is probably due to the fact that Alt's medium contained glucose, while that of Dixon & Elliott did not include any metabolite. Furthermore, Fig. 1 shows that variations in $Q_{O_2}$ from sample to sample, and from tissue to tissue occur within the cyanide-sensitive part of the respiration alone. This may be accounted for by differences in the metabolite content of the various samples and tissues, due to specific handling or the state of the nutrition of the original animal. Thus, it is likely that a liver slice (for example) taken from a rat with a high blood-sugar content would respire at a higher rate (per gram) than would a liver slice from a temporarily starved animal. These two samples would therefore exhibit divergent percentage sensitivity to cyanide, but identical cyanide-insensitive respiration (i.e. in absolute values). Similar effects might be caused by different lengths of time of soaking previous to respiration measurements.

It is clear, therefore, that *a statement of average percentage inhibition produced by cyanide does not indicate the relative activity of the cyanide-sensitive and cyanide-stable systems*. Hence the conclusion that one-third of the respiration of mammalian tissues is carried by cyanide-stable systems is by no means a valid one. On the contrary, Fig. 1 shows that when the tissue is supplied with glucose, and the cyanide-sensitive system thereby permitted to function at its maximum capacity, a total (KCN-free) $Q_{O_2}$ of about $-19$ is obtained while the cyanide-stable $Q_{O_2}$ is only $-1 \cdot 0$. Thus, *about* 95 % *of the respiration of a substrate-saturated tissue appears to be cyanide-sensitive*.

The following survey of the available data of this type serves to substantiate and extend these conclusions.

### (1) *Mammalian tissues*

Bruno Kisch (1933 *a*, *b*) has published a large amount of data on the effect of cyanide on the respiration of various mammalian tissue slices in a number of different media. The tissue slices were prepared in the conventional manner and respiration measurements were made in Warburg respirometers. Kisch presents his data in the form of a table of values of original $Q_{O_2}$ and $Q_{O_2}$ in the presence of cyanide (symbol: $Q_{O_2}^{CN}$). These data have been recalculated and plotted as graphs of original cyanide-free $Q_{O_2}$ ($y$) against the $Q_{O_2}$ inhibited by cyanide ($x$). The data for the various tissues are plotted separately, each point representing the values for the respiration of a single tissue slice. All available data are plotted in Figs. 2–5.

These show that the relationship described above holds for each of the tissues investigated. Figs. 3–5 show that the addition of carbohydrate substrate raises the original $Q_{O_2}$, but does not affect the value of $Q_{O_2}^{CN}$. On the other hand, if an amino-acid is added to the medium, the value of $Q_{O_2}^{CN}$ is increased along with the original $Q_{O_2}$. Thus, it is indicated that the respiratory breakdown of these amino-acids is

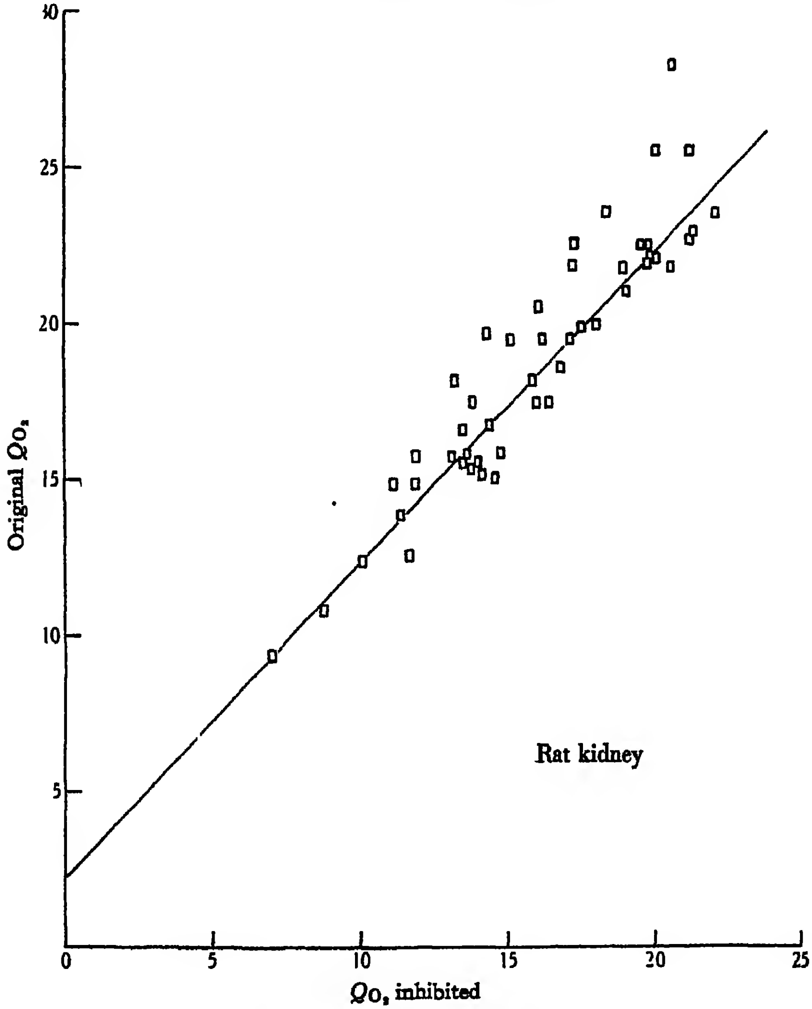


Fig. 2. Data adapted from Kisch (1933).

in part mediated through the cyanide-insensitive systems. Fig. 2 shows that the presence of phosphate in the medium (containing no metabolite) results in a similar dependence of $Q_{O_2}$ and $Q_{O_2}^{CN}$. Apart from these exceptions, the data show that differences in the $Q_{O_2}$ of different samples of the same tissue occur mainly within the cyanide-sensitive system. Further, the differences in original $Q_{O_2}$ between the various tissues are also in the main limited to variations in the cyanide-sensitive part of the respiration. Thus, while all tissues have a $Q_{O_2}^{CN}$ of about $-1{\cdot}5$, the values

of $Q_{O_2}$ (maximum) vary from $-15{\cdot}0$ (liver) to $-28{\cdot}5$ (kidney). Brain tissue appears to be quite exceptional in having a $Q_{O_2}^{ON}$ of 0.

Similar data obtained by other workers, although less extensive, show the same effect. Thus, v. Heyningen's (1935) measurements on tissue slices of liver, kidney, spleen, intestine, brain cortex, and testes of sheep, ox, rat and guinea-pig—with or without added substrate (glucose and lactose), fall on the usual straight line (see Fig. 6). A single determination, made in a medium containing pyruvate (using a

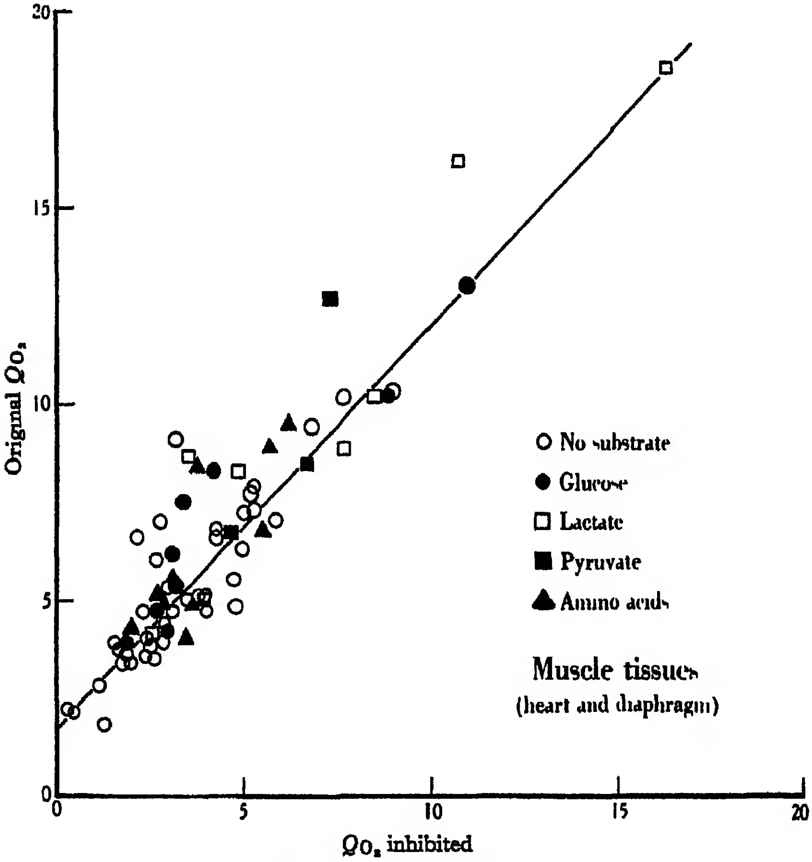


Fig. 3. Data adapted from Kisch (1933).

guinea-pig kidney slice) is exceptional in indicating a large proportion of cyanide-insensitive respiration. The values obtained for *percentage* inhibition vary from 61 to 90%, with an average of 80%.

The data obtained by Torres (1935) with rat liver, kidney, testis, brain and diaphragm respiring in Ringer's solution and in serum are also shown in Fig. 6. They follow the same relationships.

Figs. 9 and 10 show the same effect for the data obtained by Groen & Schuyl (1938) with rat liver and kidney in a non-nutrient medium.

Less complete data show similar results. Thus, Green & Brosteaux (1936),

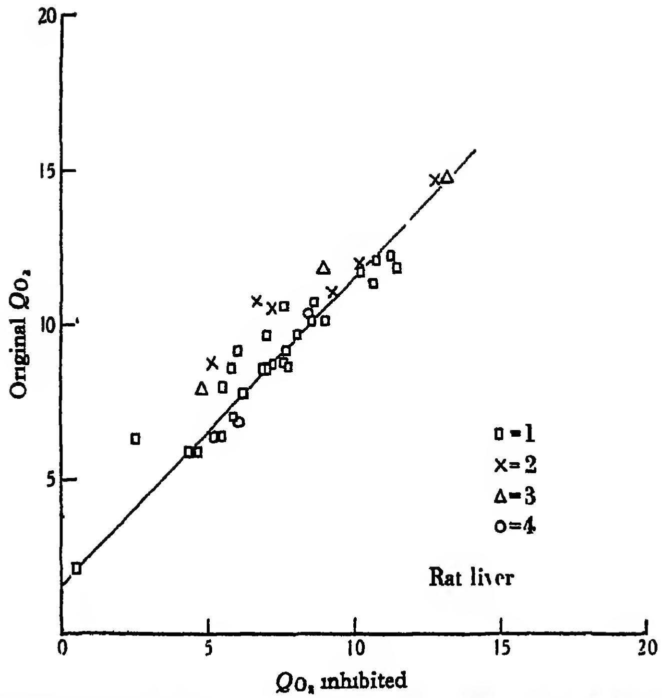


Fig. 4. Data adapted from Kisch (1933). Media used as follows: (1) no substrate; (2) amino-acids; (3) lactate; (4) glucose.

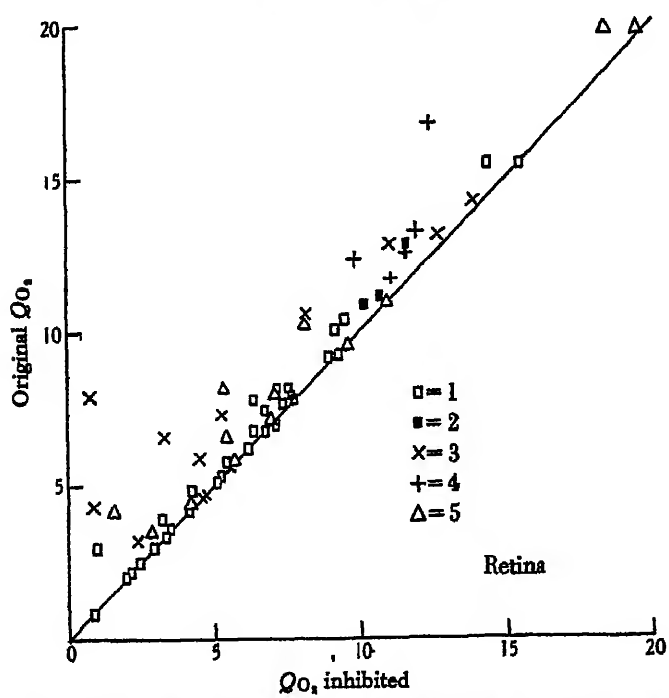


Fig. 5. Data adapted from Kisch (1933). Media used as follows: (1) no substrate; (2) glucose; (3) amino-acids; (4) lactate or pyruvate; (5) no substrate but containing phosphate.

working with rabbit brain slices, in a non-nutrient medium, obtained a $Q_{O_2}$ of $-5{\cdot}9$ and a $Q_{O_2}^{CN}$ of $-1{\cdot}3$. If the medium contained lactate ($M/125$) as a substrate, the $Q_{O_2}$ was $-10{\cdot}9$, but the value of $Q_{O_2}^{CN}$ only $-1{\cdot}7$. Thus, the increase in re-

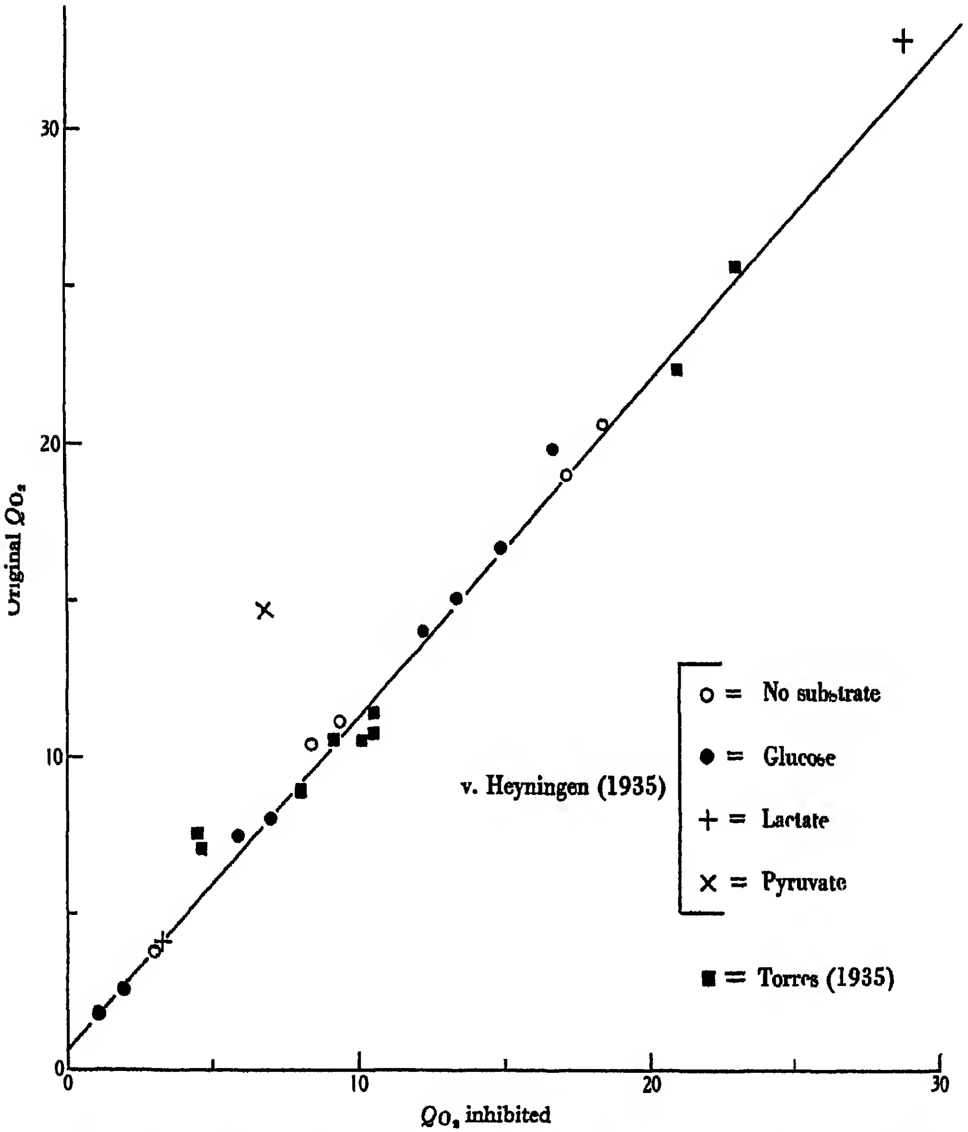


**Fig. 6. Data adapted from v. Heyningen (1935) and Torres (1935). The medium used by the latter contained no substrate.**

spiration caused by the addition of lactate to the medium was mediated through the cyanide-sensitive respiratory system alone. The same effect was found with pigeon kidney.

Similarly, Chapheau (1932) finds that the $Q_{O_2}$'s of oyster tissues in a non-

nutrient medium are: muscle, −0·21; mantle, −0·90; gills, −1·78; hepatopancreas, −1·81. Yet the values for $Q_{O_2}^{CN}$ are respectively: −0·21, −0·50, −0·095, −0·20.

These results are summarized in Table I. This table indicates that variations in $Q_{O_2}$ between the various tissues listed occur almost completely within the cyanide-sensitive system alone. Although the various values of $Q_{O_2}$ cover a wide range, the corresponding values of $Q_{O_2}^{CN}$ remain remarkably constant.

The literature reveals no significant exception to the above phenomena. Muntwyler & Binns (1933) have also made some measurements of the cyanide-sensitivity of various rat tissues in Ringer's solution. Since the medium did not contain a metabolite, the percentage inhibitions are low and range from 36 to 74 %. However, since no absolute rates are given, it is not possible to obtain any further information by means of the type of plotting described above. Stare & Elvehjem (1933), working with a wide variety of normal and specially treated mammalian tissues, also find low percentage inhibitions ranging from 42 to 72 %. However, the data for any single tissue are insufficient for plotting. The respiration of holozoic protozoa has been consistently found to be cyanide-stable (see Lund, 1918; Shoup & Boykin, 1931). This is probably due to the fact that these forms do not oxidize carbohydrates very actively and are characterized by a protein metabolism.

### (2) *Plant tissues*

The early measurements made by Warburg (1927) on the effect of cyanide on the respiration of bakers' yeast, in the presence and absence of dextrose, showed that the increase in $Q_{O_2}$ that normally follows the addition of sugar (to a non-nutrient medium) is entirely inhibited by the presence of cyanide. The value of $Q_{O_2}^{CN}$ is independent of the normal $Q_{O_2}$ and is but 5 % of the maximum value of the latter (i.e. in the presence of a maximal sugar concentration). Consequently, the percentage inhibition depends on the original $Q_{O_2}$ and thus on the dextrose concentration of the medium (since the $Q_{O_2}$ is a function of the dextrose concentration). This effect has been more completely elucidated by Commoner (1939), and certain of its aspects are further discussed below.

As noted above, Warburg (1919) also found that the respiration of *Chlorella* in a non-nutrient medium is not sensitive to cyanide. Emerson (1927*a*, *b*) found, however, that the addition of dextrose to the medium results in a four-fold increase in $Q_{O_2}$; and this increase is entirely inhibited by cyanide. Similarly, Genevois (1927*a*, *b*, 1929) has shown that the increase in the respiration rate of *Chlorella* brought about by the addition of dextrose, laevulose, mannose, galactose and various aldehydes and fatty acids is completely inhibited by cyanide. Similar results were obtained with *Scenedesmus*. These data are listed in Table I.

Genevois's determinations of the cyanide sensitivity of *Lathyrus* embryos provide evidence of a new type. The measurements were made in a non-nutrient medium, using whole embryos of various ages, etiolated embryos, starved embryos, and various parts of normal embryos. In every case the $Q_{O_2}^{CN}$ was found to be about −1·0. However, the original $Q_{O_2}$'s for the various materials varied from −1·0 to

Table I

| Tissue and organism | | Maximum $-Q_{O_2}$ | $-Q_{O_2}^{CN}$ | Respirable substrate | Reference |
|---|---|---|---|---|---|
| Kidney | Rat | 19·4 | 0·8 | + | Alt (1930) |
| ,, | ,, | 22·3 | 2·0 | − | Torres (1935) |
| ,, | ,, | 28·4 | 2·0 | + | Kisch (1933*b*) |
| ,, | ,, | 33·0 | 2·5 | + | v. Heyningen (1935) |
| ,, | Sheep | 14·0 | 1·8 | + | ,, |
| ,, | Guinea-pig | 16·7 | 2·0 | + | ,, |
| ,, | Pigeon | 18·4 | 2·8 | + | Green & Brosteaux (1936) |
| ,, | Rabbit | 5·5 | 1·2 | − | Dixon & Elliott (1929) |
| Liver | Rat | 19·0 | 1·7 | + | Kisch (1933*b*) |
| ,, | ,, | 11·1 | 2·0 | + | v. Heyningen (1935) |
| ,, | ,, | 15·1 | 0·2 | + | Alt (1930) |
| ,, | ,, | 11·4 | 0·5 | − | Torres (1935) |
| ,, | ,, | 4·3 | 1·0 | − | Dixon & Elliott (1929) |
| ,, | Sheep | 2·6 | 0·6 | + | v. Heyningen (1935) |
| ,, | ,, | 9·0 | 0·8 | − | Dixon & Elliott (1929) |
| ,, | Ox | 1·8 | 0·7 | + | v. Heyningen (1935) |
| ,, | ,, | 2·4 | 0·8 | − | Dixon & Elliott (1929) |
| ,, | Rabbit | 9·0 | 0·8 | − | ,, |
| Heart | Rat | 18·5 | 1·5 | + | Kisch (1933*b*) |
| ,, | Ox | 7·3 | 1·2 | − | Dixon & Elliott (1929) |
| ,, | Sheep | 1·2 | 0·7 | − | ,, |
| Diaphragm | Rat | 10·2 | 1·5 | + | Kisch (1933*b*) |
| ,, | ,, | 7·5 | 3·0 | − | Torres (1935) |
| Muscle | Rabbit | 1·1 | 0·3 | − | Dixon & Elliott (1929) |
| Retina | Cow | 19·8 | 0·0 | + | Kisch (1933*b*) |
| Brain | Rat | 10·8 | 0·3 | − | Torres (1935) |
| ,, | ,, | 15·0 | 1·6 | + | v. Heyningen (1935) |
| ,, | Rabbit | 3·5 | 0·3 | − | Dixon & Elliott (1929) |
| ,, | ,, | 10·9 | 1·5 | + | Green & Brosteaux (1936) |
| ,, | Sheep | 3·0 | 0·5 | − | Dixon & Elliott (1929) |
| ,, | Ox | 1·5 | 0·4 | − | ,, |
| Spleen | Rat | 12·3 | 0·6 | + | Alt (1930) |
| ,, | Ox | 4·1 | 0·8 | + | v. Heyningen (1935) |
| Intestine | Rat | 7·5 | 1·6 | + | ,, |
| Testis | ,, | 8·0 | 1·0 | + | ,, |
| ,, | ,, | 10·6 | 0·9 | − | Torres (1935) |
| Thymus | Sheep | 0·3 | 0·1 | − | Dixon & Elliott (1929) |
| Mantle | Oyster | 0·9 | 0·2 | − | Chapheau (1932) |
| Gills | ,, | 1·8 | 0·5 | − | ,, |
| Muscle | ,, | 0·2 | 0·1 | − | ,, |
| Hepatopancreas | ,, | 1·8 | 0·2 | − | ,, |
| | *Chlorella* | 5·8 | 1·2 | + | Genevois (1927*a*, *b*) |
| | *Scenedesmus* | 6·6 | 0·5 | + | ,, |
| | *Hematococcus* | 5·0 | 2·4 | + | ,, |
| | *Coleastrum* | 6·0 | 3·3 | + | ,, |
| 4-day-old *Lathyrus* embryo | | 4·3 | 1·1 | − | ,, |
| 10-day-old *Lathyrus* embryo | | 3·0 | 1·0 | − | ,, |
| Etiolated *Lathyrus* embryo | | 1·3 | 1·0 | − | ,, |
| Leaf of *Lathyrus* embryo | | 1·7 | 0·6 | − | ,, |
| Stalk of *Lathyrus* embryo | | 1·6 | 1·0 | − | ,, |
| Root of *Lathyrus* embryo | | 1·5 | 0·8 | − | ,, |
| Starved *Lathyrus* embryo | | 0·8 | 0·8 | − | ,, |
| Starved *Lathyrus* embryo | | 2·2 | 0·8 | + | ,, |
| Activated spores of *Neurospora tetrasperma* | | 10·9 | 0·3 | − | Goddard & Smith (1938) |

[1] The figures for bacteria and yeast are excluded from this table since the $Q_{O_2}$'s are not at al[l] comparable to those listed above. The values are extremely high (e.g. the $Q_{O_2}$ of bakers' yeast is o[f] the order of −100) and depend in the main upon the method used to obtain the dry weight. Since these forms are usually used in suspensions, the dry weight is rather unreliable.

$-4.3$. Thus, differences in $Q_{O_2}$ that depend on age or nutritional state occur only within the cyanide-sensitive system. This is also true for differences between the normal $Q_{O_2}$'s of various parts of the plants such as leaf, root and stalk. Similarly, Bonner (1934) reports that the percentage inhibition (by cyanide) of the respiration of *Avena* coleoptiles increases with the original $Q_{O_2}$ as the latter varies with age.

The cyanide-sensitivity of the respiration of bacteria is also conditioned by the original respiratory rate in the absence of cyanide. Thus, Gerard (1931) reports that the percentage inhibition of the respiration of *Sarcina* is greater in a medium containing glucose than in a non-nutrient medium. Burnet (1927) has shown that the bacteria whose growth is inhibited by cyanide have a relatively high aerobic respiration and are characterized by a carbohydrate metabolism. The group that was unaffected by cyanide included the streptococci and pneumococci, both of which have a relatively low rate of respiration and tend to convert glucose to lactic acid (by fermentation). Fujita & Kodama (1934) examined a large number of bacteria and found that only the actively respiring aerobes were markedly sensitive to cyanide (i.e. showed a high percentage inhibition). In particular, such active oxidizers of carbohydrates as *B. coli*, *B. dysenteriae* and *B. typhosus* were inhibited to the extent of 90%. (Unfortunately, no direct comparisons of the values of $Q_{O_2}$ and $Q_{O_2}^{CN}$ can be made since the data include only values for percentage inhibition.) On the other hand, the respiration of strict and (microaerophilic) facultative anaerobes (which have a low $Q_{O_2}$ and tend to ferment rather than respire glucose), such as *Cl. histolytica*, *Cl. welchii*, *Pneumococcus* and *Streptococcus*, is not at all affected by cyanide. Furthermore, the latter group is characterized by a lack of cytochrome while cytochrome is always present in the actively aerobic forms (see Frei *et al.* 1934).

It appears from these data that the phylogenetic variation of respiratory activity among the bacteria is due to a greater or less activity of the cyanide-sensitive system, and that this system seems to be particularly concerned with the oxidation of carbohydrates.[1] The cyanide-stable respiratory system seems to be about equally active in all forms (i.e. $Q_{O_2}^{CN}$ is about equal for all forms). It carries all of the oxidative activity in the microaerophilic forms. The higher $Q_{O_2}$ of the actively aerobic bacteria is due to the activity of the cyanide-sensitive system, and the cyanide-stable respiration is but a small part of the total. Furthermore, since most of the microaerophilic groups are characterized by a fermentative and predominantly protein metabolism, it may be suggested that the cyanide-stable system is mainly concerned with the oxidative degradation of proteins rather than with carbohydrates.

### (3) *The relative activity of the cyanide-sensitive and cyanide-stable respiration during development*

The respiratory rate of developing plants and animals has frequently been shown to vary during the course of embryogeny. The inhibitory effectiveness of cyanide

[1] Bertho & Glück (1932) show that the respiration of *B. acidophilus* in glucose is not cyanide-sensitive. The response to glucose is small, however. The increase in $Q_{O_2}$ occasioned by the addition of glucose is but 50% of that exhibited by *B. coli* (for example) under the same conditions.

has also been found to vary with the developmental state, and this can now be correlated with the changes in original $Q_{O_2}$.

The work on marine eggs illustrates this point. Warburg (1908) and others have demonstrated the rapid increase in respiratory rate that follows the fertilization of echinoderm eggs. Runnstrom (1930) has also found that the percentage inhibition of the respiration of these eggs (by cyanide and carbon monoxide) is markedly increased at fertilization. He also provides evidence to show that the increase in $Q_{O_2}$ upon fertilization is due to the sudden activation of the cyanide-sensitive system. Korr (1937) also concludes that the fertilization of *Arbacia* eggs is occasioned by the activation of the indophenol oxidase-cytochrome system. Certain echinoderm eggs do not exhibit a change in $Q_{O_2}$ upon fertilization; in others there is even a marked decrease in respiratory rate (see Whitaker, 1933). The effect of cyanide upon the respiration of such eggs has not as yet been determined, but it would be of great interest to do so. It is quite clear, however, that the increase in respiration on the fertilization of such eggs as that of *Arbacia* is mediated through the cyanide-sensitive system alone.

The effect of cyanide on the respiration of grasshopper embryos (*Melanoplus differentialis*) during the various developmental stages has been investigated by Bodine and his co-workers. They find (Bodine & Boell, 1934) that the total $Q_{O_2}$ of the embryos increases during the first 20 days of development and falls to a minimum at 40 days. This low level is maintained for the period of the diapause (from the 40th to the 90th day) during which time the developmental processes are suspended or "blocked". At the end of this period developmental activities recommence and the $Q_{O_2}$ rises sharply. The respiratory rate continues to rise from the 90th day until the embryo hatches out. During the entire course of development the value of $Q_{O_2}^{CN}$ remains constant, and is equal to the value that the $Q_{O_2}$ reaches during the diapause. Consequently, the percentage inhibition caused by cyanide is high during the pre-diapause and post-diapause periods, but falls to a value of almost zero during the diapause. Thus, it is apparent that the changes in $Q_{O_2}$ that occur during development are due to variations in the activity of the cyanide-sensitive system alone.

The data of Goddard & Smith (1938) on the increase in respiration that accompanies the activation and germination of the spores of *Neurospora tetrasperma* (a fungus) show the same effect. The dormant spores have a $Q_{O_2}$ of $-0{\cdot}27$, while activated spores that are about to germinate have a $Q_{O_2}$ of $-10{\cdot}86$. If 0·01 *M* KCN is added, the $Q_{O_2}^{CN}$ of the dormant spores is $-0{\cdot}17$, while that of the activated spores is $-0{\cdot}30$. That is, the increase in respiration that occurs upon activation is mediated almost entirely through the cyanide-sensitive system. The cyanide-sensitive respiration of activated spores is some 40 times greater than the cyanide-stable respiration, and the latter is equivalent to the total respiration of the dormant spores.

Thus, there appears to be a common pattern in the respiratory changes that accompany these cases of embryogeny, namely that variations in respiratory rate occur mainly through changes in the activity of the cyanide-sensitive system. The

cyanide-stable respiration remains at a small but constant level throughout the course of development. The precise manner in which these changes occur is by no means clear. In the case of *Arbacia* eggs, Runnström (1930) suggests that the increased activity of the cyanide-sensitive system is due to the release of "unavailable" metabolite substrates as a result of the protoplasmic activation that accompanies fertilization. On the other hand, Korr (1937) maintains that the important factor in this phenomenon is the release (upon fertilization) of the egg's cytochrome from some "unavailable" form. It may be noted here that there have been no direct investigations of the effect of the addition of various substrates on the course of these respiratory changes. Such experiments may yield important information concerning these effects.

### (4) *Respiratory gradients*

The theory of axial gradients of metabolic activity developed by Child (for a general reference see Child, 1929) and his co-workers, provides certain indirect but suggestive evidence on the inhibitory effect of cyanide. These workers have shown that many organisms exhibit an axial gradation of respiratory rate, which they suggest as the basis of the polarity and "dominance" in development and regeneration. For example, Child & Hyman (1926) have shown that sections of the hydroid *Corymorpha*, excised at various levels along its longitudinal axis, exhibit a graded intensity of respiration. The rate is at a maximum at the anterior end and decreases posteriorly. Similar determinations have shown the same to be true for a number of different forms including flat-worms, annelids, sponges, ascidians and vertebrate embryos. Furthermore, it has been found that these animals show a similarly graded susceptibility to killing by cyanide. The anterior end disintegrates most rapidly in the presence of cyanide, and the regions of lower respiratory rate do so more slowly—susceptibility being roughly directly proportional to respiratory activity. Unfortunately, there appear to be no comparable measurements of the cyanide-sensitivity of the respiration of these forms, but it would seem likely that this is directly related to the susceptibility (i.e. tendency to disintegrate) to cyanide. If so, it is suggested that the gradation of respiratory rate is in the main a consequence of variation in the activity of the cyanide-sensitive respiratory system.

### (5) *Other factors*

According to an abstract published by Boell (1938), the percentage inhibition (by cyanide) of the respiration of grasshopper embryos is proportional to the original $Q_{O_2}$ when the latter is altered by variations in temperature or in the oxygen content of the atmosphere (unpublished data). In other words, variations in the $Q_{O_2}$ that occur as a consequence of changes in temperature or $p_{O_2}$ seem to be limited to the cyanide-sensitive system. It would appear, therefore, that the cyanide-sensitive system is more labile (in the face of changes in the above conditions) than the cyanide-stable system. Korr (1937) also reports that the cyanide-sensitivity of *Arbacia* eggs increases when the $Q_{O_2}$ increases as a consequence of a rise in temperature.

(6) *Summary*

In summary we can now elucidate certain aspects of the effect of cyanide on cellular respiration.

(1) It is apparent from Table I that when the total $Q_{O_2}$ is varied by alteration in any one of a number of external factors, the absolute value of the respiratory rate in the presence of cyanide (i.e. $Q_{O_2}^{CN}$) remains remarkably constant. Thus, these variations in total $Q_{O_2}$ occur almost entirely within the cyanide-sensitive system alone. Regardless of the value of $Q_{O_2}$, the addition of cyanide (in a maximal concentration) brings the respiratory rate to the same low level. The factors which affect the $Q_{O_2}$ by altering almost exclusively the activity of the cyanide-sensitive system are:

(*a*) The concentration of available carbohydrate substrate.

(*b*) The presence of phosphate in the medium.

(*c*) The temperature.

(*d*) The concentration of oxygen in the medium $-p_{O_2}$.

(*e*) Embryonic stage.

(*f*) The particular nature of the organ (of a given organism).

(*g*) The particular nature of the species (within a phylogenetic series, e.g. bacteria).

(*h*) Random variation of samples.

The last four factors (*e*, *f*, *g*, and *h*) are, of course, more or less superficial in that they can probably be reduced to variations in the first group. However, since no such direct correlations are possible from the data available, they are listed in this form.

(2) Consequently, the percentage of inhibition produced by cyanide depends on the original rate of respiration, and with a very high $Q_{O_2}$ may approach a value of 100%. A very wide variation in percentage inhibition can be obtained merely by varying the original $Q_{O_2}$. Now, since the value of the $Q_{O_2}$ depends on the factors enumerated above, the percentage inhibition will depend on these factors as well, and is by no means a constant characteristic of a given tissue or organism. In other words, percentage inhibition of respiration by cyanide is not in itself a valid measure of the relative activity of the cyanide-sensitive and cyanide-stable systems.

(3) From the facts that most variations in $Q_{O_2}$ occur within the cyanide-sensitive system alone, and that the value of $Q_{O_2}^{CN}$ is usually but 5–10% of the possible (i.e. maximum) total $Q_{O_2}$, it becomes apparent that the cyanide-sensitive system is by far the more active. It has the greater potential respiratory capacity, and responds more readily to variations in a number of physiological factors. It is also the more labile to variations in temperature and $p_{O_2}$.

(4) It is indicated by the remarkable constancy of the values of $Q_{O_2}^{CN}$, that the cyanide-stable respiration is intimately associated with some very common property of all cells. It appears to be equally active (per unit of dry weight of protoplasm) throughout a number of different groups of organisms and tissue, and it remains constant during a number of physiological changes. In a few cases this system

carries almost all of the total respiration (which is then relatively low in value), but more often there is present a far more active and *labile* system which is inhibited by cyanide. It also seems likely that the cyanide-stable system is concerned with the oxidation of non-carbohydrate metabolites, whereas the cyanide-sensitive system is concerned with the oxidation of carbohydrate substrates exclusively.

## III. THE CYANIDE-SENSITIVE RESPIRATORY SYSTEM

The previous section has shown that the typical aerobic cell utilizes oxygen through several channels. A small, fixed part of the respiration is not affected by cyanide, while the cyanide-sensitive portion has a large and variable capacity for oxygen consumption. The relationship between these two systems has been demon-

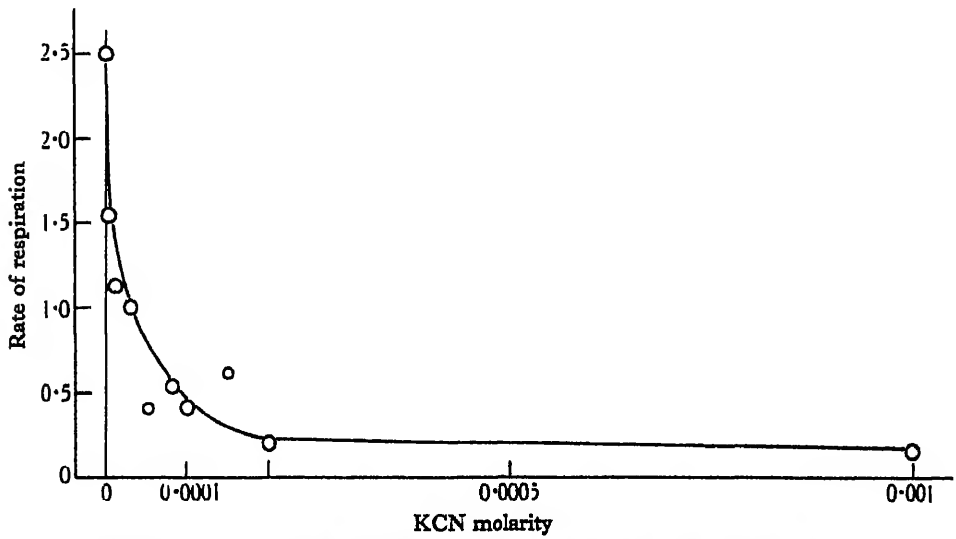


Fig. 7. The relation between the rate of respiration of bakers' yeast and the concentration of KCN in the medium (with a maximal concentration of dextrose). Reproduced from Commoner (1939).

strated above by means of an analysis of data involving the *complete* inhibition of the cyanide-sensitive system. This is done by adding cyanide in a concentration sufficient to combine with and thereby inactivate *all* of the oxidase present in the cell. However, lesser amounts of cyanide will combine with only part of the cell's oxidase, and thus the cyanide-sensitive system is only *partially* inhibited.

Consequently, by altering the concentration of cyanide in the medium, it is possible to inactivate a greater or lesser part of the cyanide-sensitive Warburg-Keilin system. The relationship between respiratory rate and cyanide concentration has been worked out in a number of cases, for example: for *Chlorella*, Genevois (1929); *B. coli*, Cook *et al.* (1931); mammalian tissues, v. Heyningen (1935); snail tissues, Baldwin (1938); grasshopper embryos, Robbie *et al.* (1938); bakers' yeast, Commoner (1939). A typical curve of this sort is shown in Fig. 7.

We have seen that *total* inhibition of the Warburg-Keilin system by means of

cyanide is a useful method of demonstrating the relationship between the cyanide-sensitive and the cyanide-stable parts of cellular respiration. Now it is also possible, by means of the *partial* cyanide inhibition, to reveal certain intrinsic properties of the Warburg-Keilin system itself.

The oxidative activity of the Warburg-Keilin system is a function of two *external* factors, namely, the concentration of metabolic substrate available, and the concentration of oxygen in the surrounding medium ($p_{O_2}$). The substrate concentration is readily altered by controlling its concentration in the medium. When this is done, it is typically found that the rate of respiration is related to the substrate concentration by a hyperbolic function characteristic of adsorption processes (see, for example, top curve of Fig. 8). The rate rises rapidly with the

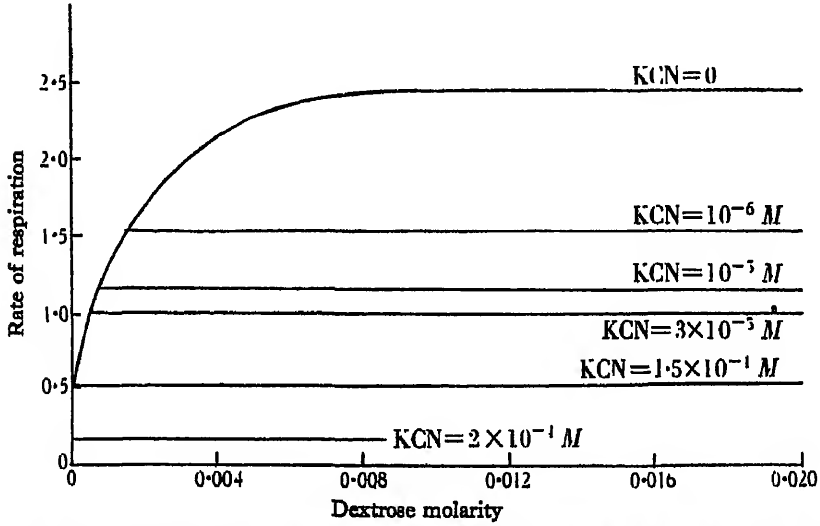


Fig. 8. The rate of respiration of bakers' yeast in various concentrations of dextrose and KCN; taken from Commoner (1939).

first increases in the substrate concentration, but finally levels off and reaches a maximum value which is maintained in spite of further addition of substrate. This phenomenon is a consequence of the limited capacity of the dehydrogenase. As the substrate concentration is increased, the "surface" of the dehydrogenase becomes increasingly saturated (with a corresponding increase in $Q_{O_2}$) until it is completely "covered" by the adsorbed substrate. Any further increase in substrate concentration will not affect the $Q_{O_2}$.

The relation between $p_{O_2}$ and $Q_{O_2}$ seems to be similarly controlled by an adsorption function; i.e. the adsorption of molecular oxygen on the oxidase (which also has a limited capacity). Because of technical difficulties, this relationship has been only partially investigated (see Tang (1933) and Kempner (1937) for summaries). A more simple method of accomplishing this task is the elimination of more or less of the active oxidase by means of cyanide.

Thus, by altering the substrate and cyanide concentrations in the medium, it is

possible to regulate the extent of the activity of the dehydrogenase and oxidase respectively. In this way, one can determine the relationship between the rate of the respiration mediated through the Warburg-Keilin system, and the two external factors that control its activity.

This relationship has been described by Commoner (1939) for the case of bakers' yeast. The data are presented in the form of Fig. 8. In submaximal concentrations of cyanide (e.g. $10^{-6} M$), the respiration is not inhibited until a critical concentration of dextrose (the substrate) is reached ($0{\cdot}002\ M$). At this point the rate ceases to increase with the dextrose concentration. Since the respiration rate in a cyanide-free medium continues to rise with the dextrose concentration even beyond this point (see upper curve of Fig. 8), the cyanide then becomes inhibitory in that it prevents this increase in rate. Thus, $10^{-6} M$ cyanide does not inhibit the respiration unless the medium contains dextrose in a concentration greater than $0{\cdot}002 M$ (i.e. the critical concentration). As the cyanide concentration is increased, the critical dextrose concentration becomes smaller. In a maximal concentration of cyanide ($2 \times 10^{-4} M$), even the endogenous respiration (i.e. in a sugar-free medium) is inhibited. Thus, the percentage inhibition produced by a given submaximal concentration of cyanide depends on the dextrose concentration. This, in a way, is only another case of the generalization made above, that percentage inhibition is a function of the original cyanide-free rate of respiration. However, in this case the effect is due to the intrinsic properties of the Warburg-Keilin system alone.

It appears from these data that the relationship between the extent of saturation of the oxidase (as controlled by the cyanide concentration), the extent of saturation of the dehydrogenase (as controlled by the dextrose concentration) and the rate of respiration, is not a continuous one. When the dehydrogenase is only slightly saturated with dextrose, a decrease in the extent of saturation of the oxidase (by the addition of cyanide) has no effect on the rate of respiration. Conversely, when the oxidase saturation is low, an increase in the saturation of the dehydrogenase (by the addition of dextrose) is of no effect. It is apparent that changes in the saturation of either the dehydrogenase or the oxidase have a discontinuous effect on the rate of respiration. This suggests that these parts of the Warburg-Keilin system are not in a kinetic homogeneous equilibrium. In this way, partial inhibition of cellular respiration by cyanide provides a description of certain internal properties of the Warburg-Keilin system.

An extension of this phenomenon can be found in the work of other authors. In the case cited above, we dealt with but a single dehydrogenase. Actually, as Quastel & Whetham (1925*a*, *b*) have shown, the oxidation of each carbohydrate substrate is mediated through a dehydrogenase specific to it. Consequently, we may think of the Warburg-Keilin system of a typical cell as mediating the oxidation of several carbohydrate substrates through as many separate dehydrogenase enzymes. Thus, the cyanide sensitivity of the oxidation of a number of such substrates (and various mixtures of these) can provide further information concerning the internal relationships of this respiratory system.

This has been done by Cook *et al.* (1931) and by Cook & Haldane (1931). These authors show that the $Q_{O_2}$ of toluene-treated *B. coli* varies with the particular carbohydrate present in the medium. Thus, for equivalent amounts of each substrate, the $Q_{O_2}$'s obtained are: for formate, −5571; lactate, −1723; succinate, −1277; glucose, −855; no substrate, −217. It was also found that the sensitivity of the respiration of the bacteria to cyanide and carbon monoxide increased with the $Q_{O_2}$ and was thereby dependent on the nature of the substrate. Thus, 0·01 $M$ cyanide caused an inhibition of 98·4% if formate was present, but only a 61% inhibition in the presence of lactate (in equivalent concentrations). That is, in spite of the wide divergence in the values of $Q_{O_2}$ in formate and lactate, the values of $Q_{O_2}^{CN}$ are very similar: formate, −89, and lactate, −66. The sensitivity to carbon monoxide, as determined by the value of the affinity constant $K$ [$\left(K = \dfrac{\text{affinity of oxidase for } O_2}{\text{affinity of oxidase for CO}}\right)$, i.e. small values of $K$ indicate extreme sensitivity to CO] showed similar relationships. The value of $K$ for formate was found to be 3; for succinate, 6; for lactate, 10; and for glucose, 20–27.

A similar effect was found by Ogston & Green (1935*b*) for the respiration of brewers' yeast in various substrates. As shown by Table II, the variation in $Q_{O_2}$ due to difference in substrate is (relatively) far greater than the variation of $Q_{O_2}^{CN}$.

Tamiya & Kubo (1938) find the same to be true of the respiration of *Acetobacter* in various substrates including ethyl alcohol and glucose.

In general, it appears that the cyanide (or CO) sensitivity of the respiration of these various substrates is a function of the relative capacity of its specific dehydrogenase, and is thereby related to the original $Q_{O_2}$. The dehydrogenases with the greater capacities (i.e. those producing the greater $Q_{O_2}$) require a greater degree of activity on the part of the oxidase, thus rendering the respiratory rate more sensitive to the reduction in oxidase saturation which is occasioned by the addition of cyanide.

Table II. (*From Ogston and Green* (1935*b*))

| Substrate | $Q_{O_2}$ | $Q_{O_2}^{CN}$ |
|---|---|---|
| None | 65·5 | 26·4 |
| Lactate | 570·0 | 54·0 |
| Hexose-monophosphate | 298·0 | 25·0 |
| Hexose-di-phosphate | 123·0 | 41·0 |
| Glycero-phosphate | 149·0 | 56·5 |
| Glucose | 306·0 | 65·5 |

Here, again, we have an example of the general rule relating cyanide sensitivity to the original rate of respiration.

Cook *et al.* (1931) suggest, on the basis of the differential sensitivity of the respiration of various substrates, that there are as many different oxidases as there are dehydrogenases. They differentiate these oxidases on the basis of their respective cyanide (and carbon monoxide) affinity "constants". Since these constants are defined in terms of percentage sensitivity to the poisons, their values only indicate

differences in the dehydrogenase activity and *not* the supposed differences between the several oxidases (cf. Commoner, 1939). The suggestion of multiple oxidases is not necessary in the light of the above analysis. The controlling factor is the activity of the dehydrogenase, whether this is due to its specific capacity or to the concentration of the substrate.

## IV. THE CYANIDE-STABLE RESPIRATORY SYSTEM

Since the cytochrome oxidase which is responsible for the reduction of molecular oxygen is the locus of cyanide-sensitivity, any respiratory system which is *not* cyanide-sensitive must be capable of reacting with molecular oxygen by some other means. Artificially, such systems can be readily produced by the use of methylene blue or a similar dye which is capable of reversible oxidation and reduction. Cellular enzymes such as the dehydrogenases reduce methylene blue to its leuco-base and the latter is reoxidized by molecular oxygen. The cellular components which are capable of this type of reaction have been mentioned in § I. Almost invariably, the properties of these substances have been determined by *in vitro* experiments with cell-free extracts, and it is therefore difficult to estimate their relative activity within the entire cell (i.e. as compared with the activity of the Warburg-Keilin system). Only in one case, that of the so-called "yellow enzyme", is there sufficient *in vivo* data to warrant such an estimation.

Warburg & Christian (1932) isolated from yeast a "yellow enzyme" that could be reduced by hexose-mono-phosphate and reoxidized by molecular oxygen in the presence of a coenzyme extracted from erythrocytes. It was soon shown by the work of Theorell (1935) and others, that this enzyme is composed of a protein combined with a prosthetic pigment group. The pigment was found to be identical with phosphorylated riboflavin (or lactoflavin) previously isolated from animal tissues by v. Euler & Adler (1934) and Gyorgyi *et al.* (1934). The substance was also found in yeast and bacteria by Warburg & Christian (1933) and in plants by Kuhn *et al.* (1934). The *in vitro* properties of the yellow enzyme have been summarized by Theorell (1937). He shows that the following metabolites may act as substrates of the yellow enzyme (in the presence of the proper dehydrogenase extract): Robison ester, Neuberg ester, alcohol, glucose, malic acid, lactic acid, citric acid, and hexose-di-phosphate. The oxidation of these substrates is mediated through a coenzyme which is a tri-phosphopyridine nucleotide containing nicotinic acid amide and which is combined with a protein "carrier group". From this sort of work with *extracts* of the yellow enzyme, the following respiratory system may be constructed:

$$O_2 \rightarrow Y \rightleftharpoons YH_2 \rightleftharpoons CO \rightleftharpoons COH_2 \leftarrow \text{Substrate} + \text{Dehydrogenase},$$

where $Y$ is the oxidized yellow enzyme; $YH_2$ is the reduced yellow enzyme; CO is the oxidized co-enzyme; $COH_2$ is the reduced co-enzyme. The question of the actual *in vivo* substrates of this system will be dealt with below. It can be seen at once, however, that this system permits of a cyanide-insensitive cellular respiration.

Several workers have attempted to correlate the cyanide-stable respiration ($Q_{O_2}^{CN}$) of various tissues and organisms (i.e. *in vivo*) with the yellow enzyme activity. *Lactobacillus*, for example, is known (Bertho & Gluck, 1932) to have a relatively high lactoflavin content and a respiratory rate which is low in value and stable to cyanide. Adler & v. Euler (1934) obtained a 100 % increase in the respiration

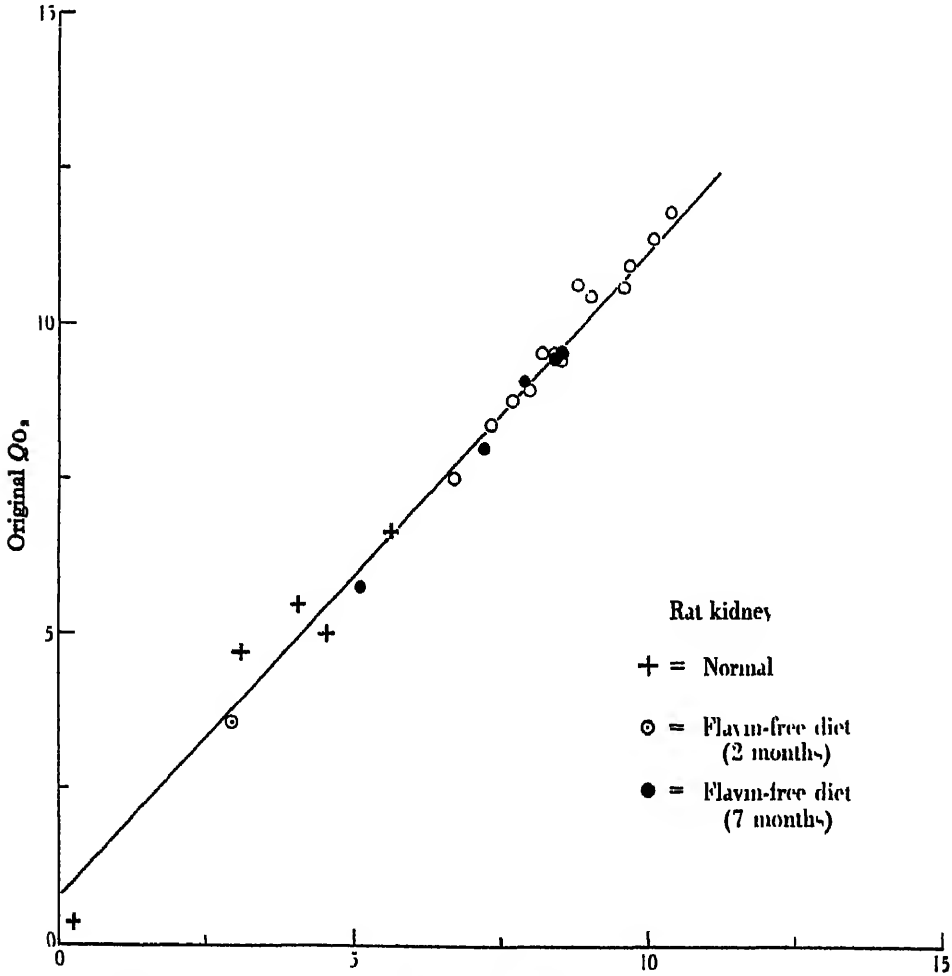


Fig. 9. The effect of a flavin-free diet on the cyanide-sensitivity of rat kidney. Data adapted from Groen & Schuyl (1938).

of *Lactobacillus* by adding lactoflavin to the culture medium. This additional respiration, like the original respiration, was not inhibited by cyanide.

Torres (1935) attempted to correlate the lactoflavin content of various tissues with the values of $Q_{O_2}^{CN}$ which they exhibited. No positive correlation was obtained. Gourévitch (1937), using an improved technique for the determination of the lactoflavin content, reports a definite relationship. This is shown in Table IV,

together with lactoflavin determinations made by v. Euler & Adler (1934). Although it is doubtful that the smaller differences in $Q_{O_2}^{CN}$ are significant, certain correlations which are based on marked variations, can be considered valid. Thus, retina tissue, which is remarkable in having a residual cyanide-stable respiration of about zero (see also Fig. 5), has the lowest lactoflavin content. On the other hand, kidney tissue is highest both in lactoflavin content and residual respiration. Gourévitch concludes that, "Cet ensemble de faits semble donc indiquer que la respiration

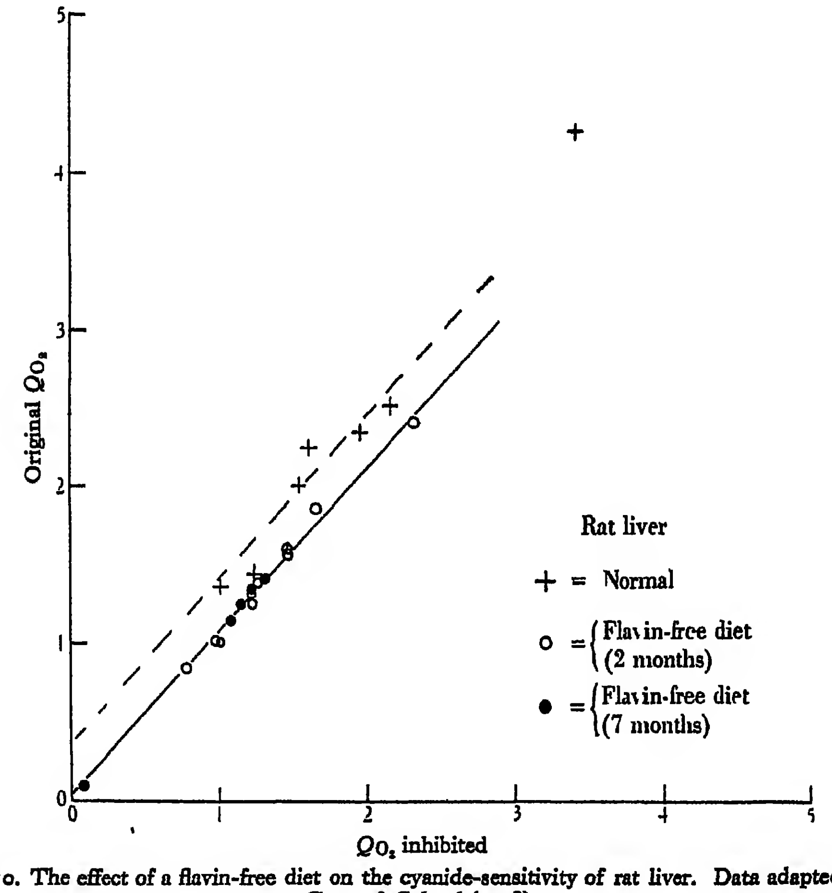


Fig. 10. The effect of a flavin-free diet on the cyanide-sensitivity of rat liver. Data adapted from Groen & Schuyl (1938).

residuelle des tissues des mammifères doit être entièrement attribué au système respiratoire flavine-ferment".

More conclusive evidence has been obtained by Groen & Schuyl (1938). They separated a common stock of rats into three groups. One group was fed on a normal diet, the second on a lactoflavin-free diet for a period of 2 months, and the third on a lactoflavin-free diet for 7 months. In each case respiration measurements of liver and kidney slices were made in Ringer's solution and in Ringer's solution plus 0·01 % NaCN. Determinations showed that the lactoflavin content of the liver fell from 10·2 $\mu$g./g. to 5·2 $\mu$g./g. after 2 months on the lactoflavin-free diet. The same

lactoflavin content was found after 7 months of this diet. On the other hand, the lactoflavin content of the kidney did not vary more than 15%. Normal kidney contained 16·8 μg./g. After 2 months of the diet, it contained 15·9 μg/g., and after 7 months 14·4 μg./g. When the respiration data are plotted in the manner used above (see Figs. 9 and 10), the relationship between the cyanide-stable respiration and the lactoflavin content is quite apparent. In all cases, we obtain the usual straight line with a 45° slope. The values obtained for normal liver tend to fall on a straight line that is raised above the level for that of the lactoflavin-free diet, but parallel to it. The intercept of the lactoflavin-free line indicates a value of $-0{\cdot}05$ for the $Q_{O_2}^{CN}$, while the line for normal liver indicates a $Q_{O_2}^{CN}$ of $-0{\cdot}4$. Thus, the loss of flavin from the liver is accompanied by a corresponding reduction in the respiratory activity which is stable to cyanide. As would be expected from the fact that the normal and treated kidney tissues have similar lactoflavin contents, the respiratory data of these two coincide (see Fig. 9). Thus, it is indicated that the absolute value of the cyanide-stable respiration ($Q_{O_2}^{CN}$) is directly related to the lactoflavin content of the tissue.

This conclusion is given further support by the finding of Pett (1935, 1936) that when yeast is cultured for 5 days in a medium containing *M*/600 KCN, its flavin content is doubled and correspondingly the value of $Q_{O_2}^{CN}$ is also doubled.

## V. THE SUBSTRATE SPECIFICITIES OF THE CYANIDE-SENSITIVE AND CYANIDE-STABLE RESPIRATORY SYSTEMS

There are some indications from the evidence cited in the earlier parts of this paper (see p. 175), that the cyanide-sensitivity of cellular respiration depends to some degree upon the particular metabolite undergoing oxidation. Several workers have suggested that this type of specificity accounts for certain of the variations in the magnitude of the cyanide-stable respiration (Keilin, 1932; Kisch, 1933*b*; Oppenheimer, 1938).

It is clear that this question is exceedingly complex. The increase in consumption of oxygen upon addition of a particular metabolite does not necessarily mean that this substance is being completely oxidized to carbon dioxide and water through the mediation of a single respiratory chain (or system). The substrate may be partially oxidized by one type of oxidative enzyme, yielding thereby a new substance which may then be oxidized by another enzyme system. However, taking the respiratory process as a whole, regardless of the kind and number of steps whereby complete oxidation is achieved, it is possible to pose the following question: Is the total oxidation of a given metabolite shared between the cyanide-sensitive and cyanide-stable respiratory systems; or does the oxidation of each metabolite tend to be mediated by one of the two systems exclusively? It is impossible, with the data at present available, to offer a definitive answer, but certain clarification of the question may be attained.

Keilin (1929, 1930) indicated that the Warburg-Keilin system is capable of reacting with many different dehydrogenases, and hence is able to oxidize many of the normally present metabolites. This view was based on the observations of the cytochrome activity of living cells in the presence of various substrates. His experi-

ments were not very extensive, but it was suggested that the cytochrome-cytochrome-oxidase system had a general oxidizing power that was applied to a number of the existing dehydrogenases, and particularly to those that mediated the oxidation of carbohydrates.

The data concerning the substrates of the yellow enzyme system are, in the main, based upon *in vitro* tests with tissue extracts of the component enzymes. It has been shown by Euler & Adler (1934) and others that extracts of the yellow enzyme and its coenzyme are capable of producing an active oxidation of hexose phosphates. The summary of Theorell (1937) lists a number of diverse metabolites as substrates (according to *in vitro* tests) of the yellow enzyme (see p. 189).

Ogston & Green (1935*a*, *b*) have conducted a number of *in vitro* experiments concerning the substrates of both the Warburg-Keilin system and the yellow enzyme system. They prepared extracts of the constituent enzymes and measured the rates of oxidation after the addition of various metabolites (with the specific dehydrogenase already present). The following substrates were tested: lactate, hexose-di-phosphate, hexose-mono-phosphate, succinate, xanthine, uric acid, glucose, malate, and alanine. Of these, the yellow enzyme system was able to oxidize the hexose phosphates, glucose and (to a lesser extent) malate, while the Warburg-Keilin system oxidized only lactate and succinate.

On the basis of these observations, Ogston & Green criticize the view of Keilin on the general nature of the oxidative properties of the Warburg-Keilin system. However, the validity of this criticism and the applicability of their results to *cellular* respiration becomes doubtful in the light of other results obtained by the same authors (Ogston & Green, 1935*b*). Here they show that the respiration of hexose-phosphates by brewers' yeast is completely cyanide-sensitive. This yeast contains a considerable quantity of yellow enzyme. When this enzyme is extracted and added to hexose-phosphate (together with the co-enzyme), the substrate is rapidly oxidized, and the rate is not affected by cyanide. Thus, in the living yeast cell the oxidation of this substrate is mediated by the cyanide-sensitive system and the yellow enzyme system is not at all active, while *in vitro* the reverse is true.

It is apparent, therefore, that the substrate specificities of *in vitro* respiratory systems are not at all descriptive of the conditions which actually obtain in the living cell. It seems necessary to restrict the evidence to *in vivo* data. Such data can be obtained by measuring the respiration of a tissue (or one-celled organism) before and after the addition of a given substrate; and then, in cases where a definite increase in rate occurs, also determining the effect of cyanide. Thus, if a given metabolite causes a marked increase in the respiration of some tissue, and if cyanide inhibits this increase, it may be concluded that this substrate is oxidized by the cyanide-sensitive (Warburg-Keilin) system. On the other hand, if cyanide does not inhibit the increase in respiration, the oxidation is carried by the cyanide-stable system. Table III summarizes the available data on the cyanide-sensitivity of the respiration of various substrates by different living cells.

This table bears out the general sense of Keilin's view, namely, that the cyanide-sensitive system oxidizes the wider range of substrates. The respiration of carbo-

Table III. A. *Substrates which increase the cyanide-sensitive respiration*

| Substrate | Tissue or organism | Reference |
|---|---|---|
| Glucose | Bakers' yeast | Warburg (1927), Commoner (1939) and others |
| „ | Brewers' yeast | Ogston & Green (1935*b*) |
| „ | *B. coli* | Cook *et al.* (1931) |
| „ | *Sarcina* | Gerard (1931) |
| „ | *Chlorella* | Genevois (1927*a*) |
| „ | *Scenedesmus* | „ |
| „ | *Lathyrus* embryos | Genevois (1927*b*) |
| „ | Kidney* | Kirsch (1933*b*) |
| „ | Liver* | „ |
| „ | Diaphragm* | „ |
| „ | Ventricle* | „ |
| „ | Retina* | „ |
| Fructose | *Chlorella* | Emerson (1927*a*, *b*) |
| Galactose | *Chlorella* | „ |
| Mannose | *Chlorella* | „ |
| Lactate | *Acetobacter* | Tamiya & Kubo (1938) |
| „ | Brewers' yeast | Ogston & Green (1935*b*) |
| „ | *B. coli* | Cook *et al* (1931) |
| „ | Brain | Green & Brosteaux (1936) |
| „ | Kidney, etc. | Kisch (1933*b*) |
| Succinate | *B. coli* | Cook et *al.* (1931) |
| Formate | *B. coli* | „ |
| Hexose-phosphates | Brewers' yeast | Ogston & Green (1935*b*) |
| Ethanol | *Acetobacter* | Tamiya & Kubo (1938) |
| Mannitol | *Azotobacter* | Negelein & Gerischer (1934) |
| Choline | Liver | Mann & Quastel (1937) |
| Acetaldehyde | *Chlorella* | Genevois (1927) |
| Butyric acid | *Chlorella* | „ |
| Serin | Kidney, etc. | Kisch (1933*b*) |
| Sarcosin | „ | „ |

B. *Substrates which increase the cyanide-stable respiration*

| Substrate | Tissue or organism | Reference |
|---|---|---|
| Lupinus oil | *Lupinus albus* | Craig (1936) |
| Pyruvate | Kidney, etc. | Kisch (1933*b*) and v. Heyningen (1935) |
| Alanine | „ | Kisch (1933*b*) |
| Glycine | *Elodea* | Schwabe (1932) |
| Glucose | *Lactobacillus* | Bertho & Gluck (1932) |

In the case of the tissues marked *, Kisch reports that the respiration of glucose and lactose is sometimes only partially sensitive to cyanide.

hydrates and short-chain hydroxy-fatty acids (lactic acid, etc.) appears to be limited to the cyanide-sensitive system. Both systems seem to respire amino acids. The single case of fat respiration is cyanide-stable. The cyanide-stability of pyruvate respiration is probably an artefact. Green & Williamson (1936) have shown that pyruvate tends to combine with cyanide to form a non-toxic cyanohydrin, thereby negating the inhibitory effect of the cyanide. The case of the cyanide-stable respiration of glucose by *Lactobacillus* provides an interesting exception. It has been shown above (p. 181) that bacteria such as *Lactobacillus*, *Streptococcus* and *Pneumococcus*, that are characterized by a relatively low rate of respiration and the conversion of glucose

to lactic acid, are not sensitive to cyanide. These forms tend to ferment rather than to oxidize most of the available glucose. It may be that this property is in some way related to the predominance of the cyanide-stable respiration.

Wright & van Alstyne (1931), Keilin (1932) and Oppenheimer (1938) suggest that the cyanide-stable respiration may be due to the oxidation of fat. This suggestion was based on certain *in vitro* data. Robinson (1924) showed that the catalysis by hemin of the oxidation of linoleic acid was unaffected by cyanide; while similar catalysis of the oxidation of carbohydrates was cyanide-sensitive. Kuhn & Meyer (1929) also showed that the hemin catalysis of the oxidation of olive oil and of such long-chain fatty compounds as bixin is not cyanide-sensitive. These results were confirmed by Wright & van Alstyne (1931). Although the validity of such results as an indication of the nature of *cellular* processes is subject to the limitations mentioned above, there is some *in vivo* evidence to support them. Craig (1936) describes the oxidation of *Lupinus* oil by the pulverized seeds of *Lupinus albus*. The effect of cyanide was variable, but generally not inhibitory (the maximum inhibition obtained was but 30 %).

This hypothesis, i.e. the substrate-specificity of the inhibition of respiration by cyanide, may be tested by an analysis of certain of the data presented in the earlier parts of this paper, together with evidence of a new sort. The crucial points which must be subjected to test are these: (1) cyanide-sensitive respiration, which is mediated through the Warburg-Keilin system, is concerned with the oxidation of carbohydrates and substances which have a similarly high ratio of oxygen to carbon (O/C) (such as hydroxy-acids); while (2) the cyanide-stable respiration is mediated through the yellow enzyme system and is concerned with the oxidation of fatty substances: i.e. compounds with a low O/C ratio and containing long $—CH_2—$ chains. Although the data are, unfortunately, rather limited, Table IV provides at least a tentative support of this hypothesis.

It is apparent that the tissues listed may be divided into two qualitatively distinct groups—one including nervous tissues (retina and brain cortex) and the other, kidney, heart and liver. Testis and spleen have intermediate characteristics.

Table IV

| Tissue | (1) $Q_{O_2}^{CN}$ | (2) Flavin content $\mu$ per g. of fresh tissue | (3) Response to glucose. % of original rate of respiration | (4) Normal respiratory quotient | (5) Respiratory quotient in the presence of cyanide |
|---|---|---|---|---|---|
| Liver | 2·0–1·5 | 17·5 | 90 | 0·79 | 0·50 |
| Kidney | 2·4–2·0 | 17·8 | 120 | 0·85 | 0·38 |
| Heart | 2·4 | 10·3 | — | 0·85 | — |
| Spleen | 0·8 | 1·0 | 120 | 0·89 | — |
| Testis | 1·0 | — | 220 | 0·94 | 0·87 |
| Retina | 0 | 0 | 550 | 1·00 | — |
| Brain | 1·6–0·3 | 0 | 320 | 0·99 | 0·97 |

The data were obtained as follows: Column (1) from the intercepts of the lines in Figs. 1–6; column (2) from Gourévitch (1937); columns (3) and (4) from Dickens & Greville (1933); and column (5) from Himwich *et al.* (1933).

Retina and brain cortex exhibit almost no cyanide-stable respiration. Furthermore, they contain an insignificant amount of flavin, and it would appear therefore that the yellow enzyme system is but slightly if at all active. These tissues show a clear specificity as to respirable substrates. They respond markedly to the addition of glucose (see column 3, Table IV). In addition, Quastel & Wheatley (1932) report that, of a wide variety of metabolites that were tested, only the following acted as respirable substrates for brain tissue: glucose, fructose, mannose, galactose, lactate, pyruvate, succinate and glutamic acid. Thus, brain tissue is characterized by the active respiration exclusively of carbohydrates and compounds with a similarly high O/C ratio. As would be expected therefrom, its respiratory quotient (R.Q.) is equal to 1·0;[1] and the R.Q. is not appreciably altered by the addition of cyanide. The same is true of retina tissue. Thus, these tissues exhibit a close correlation between predominance of the cyanide-sensitive system and utilization (exclusively) of carbohydrates and substances with a similar O/C ratio.[2]

The second group, including kidney, liver and heart tissue, shows some internal differences, but these do not appear to be sufficiently distinct to warrant a correlative analysis. Taken as a group (as compared to retina and brain cortex), however, they show certain marked correlations. These tissues have a distinct cyanide-stable respiration and all of them contain considerable quantities of flavin. We must conclude therefrom, that the cyanide-stable yellow enzyme system is more active than it is in the nervous tissues. However, the Warburg-Keilin system is also present in these tissues, since they are partially sensitive to cyanide. Column 3 indicates that the tissues of the second group are but moderately if at all responsive to glucose. On the other hand, column 4 shows that they tend to oxidize substrates with a low O/C ratio, i.e. those yielding a R.Q. less than 1·0 (e.g. fats and proteins). Furthermore, the addition of cyanide brings the R.Q. to an even lower level, thereby indicating that cyanide inhibits preferentially the oxidation of metabolites yielding a high R.Q., i.e. those with a high O/C ratio.[3]

It may be suggested therefore, that the criterion which determines the respiratory

[1] The respiratory quotient is defined as the ratio of the carbon dioxide produced to the oxygen absorbed, i.e. $CO_2/O_2$. Since the basic formula of a carbohydrate is $(CH_2O)_n$, only the carbon atoms require oxidation. Thus, each molecule of $O_2$ oxidizes one C atom, and produces one molecule of $CO_2$, thereby resulting in a R.Q. of 1. In compounds which have a low O/C ratio the hydrogen content is much greater than it is in the carbohydrates. Thus, these excess H atoms (beyond the ratio 2H/O) must be oxidized by the $O_2$, to produce $H_2O$. Consequently, several molecules of $O_2$ are consumed for each molecule of $CO_2$ produced, and the R.Q. is less than 1.

[2] It is interesting to note that nervous tissues are also characterized by an extreme sensitivity to lack of oxygen. There seems to be a close dependency between this type of sensitivity and sensitivity to cyanide. Thus, the first effects of both a lack of oxygen and of cyanide poisoning (on higher animals) is the inactivation of the brain.

[3] A value of less than 1·0 for the R.Q. may also indicate the *incomplete* oxidation of a *carbohydrate* substrate, with the production of some substance which is still oxidizable, rather than $CO_2$ and $H_2O$. However, this does not seem to be the case in the data cited in Table IV. Since the R.Q. of liver, etc., is further reduced by cyanide, the above interpretation would mean that *partial* oxidation of carbohydrates is cyanide-stable, while *complete* oxidation of the same substrate is cyanide-sensitive. Hence, were this so, we would expect that the addition of a carbohydrate such as glucose would increase *both* the $Q_{O_2}$ *and* the $Q_{O_2}^{CN}$ of these tissues. This does not occur; and it seems likely therefore that the R.Q., in this case, actually indicates the complete oxidation of metabolites with a preponderance of $—CH_2—$ groups.

path of a given substrate is its relative oxygen (usually in the form of hydroxyl) content. Substrates of the fatty type, containing extensive —$CH_2$— chains and little oxygen (and therefore yielding a low R.Q.), seem to be specific for the yellow enzyme system. Substrates of the carbohydrate type tend to be respired specifically by the Warburg-Keilin system.

Finally, the hypothesis suggested above offers a possible explanation of the extreme lability of the cyanide-sensitive respiration as compared with the cyanide-stable respiration. The substrates of the cyanide-sensitive system are characterized by a marked solubility in water. Therefore it is not surprising to find that their concentration in normal tissues is very variable—thereby accounting for the variability of the normal $Q_{O_2}$, and the fact that these variations are cyanide-sensitive (see Figs. 1–6). On the other hand, the substrates of the cyanide-stable respiration, since they contain extensive —$CH_2$— chains, are relatively insoluble. Consequently their tissue concentrations are relatively constant, thereby resulting in a rather constant value of $Q_{O_2}^{CN}$. Furthermore, the remarkable similarity of the $Q_{O_2}^{CN}$ values of various tissues and organisms (see Table I) suggests that the concentration of these substrates is related to some internal property common to all these different cells. Hence, it may not be amiss to suggest that the protein chains of the protoplasm itself may act as a constant and ever-present supply of substrate for the cyanide-stable respiration.

However, it is obvious that the available data do not permit of any definitive statement as to substrate specificities and these conclusions are offered in purely tentative terms.

## VI. SUMMARY

The effect of cyanide on the respiration of living cells makes possible the differentiation between and the description of two types of respiratory systems.

(1) The cyanide-sensitive respiration may be identified with the Warburg-Keilin respiratory system. The normal variation in $Q_{O_2}$ between different organs, organisms, developmental stages (in certain enumerated cases) and random variations among samples of the same tissue, seem largely to be accountable by differences in the activity of this system. The rate of oxygen consumption by this system varies over a wide range of values, and accounts for about 90% of the *maximum possible* respiratory rate of most actively aerobic tissues and organisms. It seems likely (on the basis of the available data) that the cyanide-sensitive system tends most actively to oxidize carbohydrates and other metabolites that have a similarly high water-solubility and O/C ratio. Thus, cyanide-sensitive respiration is characterized by a respiratory quotient of about 1·0. The Warburg-Keilin system also appears to be the more sensitive (of the two) to variations in temperature and $p_{O_2}$.

(2) The cyanide-stable respiration may be identified with the yellow enzyme or flavoprotein. Its activity is relatively small and constant from organ to organ and from organism to organism (within certain groups), as compared with the activity of the cyanide-sensitive system. It seems likely that the metabolic substrates of this

respiratory system (*in vivo*) are restricted, in the main, to fatty compounds and other substances that have a similarly low O/C ratio. Thus, the cyanide-stable respiration is characterized by a respiratory quotient of 0·8 or less.

Hence, since most variations in the total rate of respiration are mainly due to variations in the activity of the cyanide-sensitive system alone, percentage inhibition by cyanide (i.e. "cyanide-sensitivity") increases with the normal rate of respiration. Therefore, this value is no index of the relative activity of the two systems unless the substrate environment and other influential conditions are specified.

Certain intrinsic properties of the Warburg-Keilin system are elucidated by *partial* inhibition of this system. It is shown thereby that the inhibitory effect of cyanide is related to the extent of saturation of the dehydrogenase with its substrate. When the dehydrogenase is only partially saturated or "covered", the respiratory rate is reduced only by high cyanide concentrations. Thus, under such circumstances, part of the oxidase may be inactivated by a low concentration of cyanide without affecting the rate of respiration. However, such a cyanide concentration prevents the rise in respiratory rate that normally follows the restoration of the dehydrogenase to complete saturation by the addition of substrate to the medium. Thus, in this case we again note that the percentage inhibition by cyanide increases with the original (i.e. cyanide-free) rate of respiration.

It appears therefore that the respiratory systems that can be distinguished by means of cyanide-sensitivity are to a large degree independent of each other. It has frequently been suggested that the two systems present alternative paths for the oxidation of any given metabolite. The amount of oxidative activity of each system would then depend on their relative concentrations in the cell and on their relative oxidative tendencies, i.e. redox potentials. If this were so, we would expect that the ratio of their activities would be constant regardless of the nature or concentration of the metabolite present in the medium. Furthermore, if the activity of one of these systems be curtailed (e.g. by cyanide), it follows that the insensitive system would become more active since it would then be exposed to a greater portion of the "reduction potential" which is produced by the metabolite and its dehydrogenase.

It does not seem likely, therefore, that this is a valid interpretation of the interrelationships of these processes as they obtain in the living cell. The ratio of the rates of activity of the two systems (i.e. the percentage inhibition by cyanide) is not constant, but varies directly with the total rate of respiration. Thus, in a living cell the cyanide-stable system seems to operate at a low and constant rate, to a large degree independent of the highly variable rate of activity of the cyanide-sensitive system. Furthermore, the systems appear to have qualitative specificities as to substrates, rather than sharing the oxidation of each metabolite. These facts seem to indicate that there is no direct thermodynamic equilibrium between the oxidative activity of the two systems.

The internal relationships of the various parts of the cyanide-sensitive system appear to be equally discontinuous. Under certain conditions the saturation of the oxidase or the dehydrogenase may be altered without affecting the total rate of respiration.

Finally, these specific relationships of the respiratory systems are intimately associated with the normal integrity of the cell. Destruction of the cellular protoplasm disorients the qualitative specificities that obtain in the living cell. It would appear that the ordering of the interrelated processes of cellular respiration is in some manner facilitated by the normal intracellular structure.

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## ADDENDUM

Since the above review was submitted there have appeared several new data that are of importance. J. N. Stannard has published a paper entitled "Separation of the resting and activity oxygen consumptions of frog muscle by means of sodium azide" (*Amer. J. Physiol.* 1939, **126**, 196). Here it is shown that the increase in respiration that accompanies the activation of a muscle is completely inhibited by cyanide. Also Marsh and Goddard ("Respiration and fermentation in the carrot, *Daucus carota*. I. Respiration." *Amer. J. Bot.* 1939, **26**, 724) have found that the carrot tissues with the higher normal rate of respiration show a higher percentage sensitivity to cyanide. Finally Korr (private communication) finds that the increase in respiration that occurs when salivary gland is activated with acetylcholine and pancreas by secretin, is completely inhibited by cyanide.

# PALAEONTOLOGICAL EVIDENCE BEARING ON HUMAN EVOLUTION

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(*Received* 1 *August* 1939)

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## I. INTRODUCTION

For some time now there has been a general agreement regarding the broad lines of evolution which led to the emergence of man from a primate ancestry. Already in Palaeocene and Eocene times small primitive primates were in existence throughout many parts of the world. These can be subdivided into two main groups—primitive lemuroids represented by the Plesiadapidae and Adapidae, and primitive tarsioids, represented by the Anaptomorphidae and Microchoeridae. The precursors of the modern Lemuroidea became specialized very rapidly, and even in the Eocene showed divergent modifications of a characteristic kind. It is now usually admitted, indeed, that the Lemuroidea, as they are commonly defined, could hardly have had a place in the ancestry of the higher primates in which such specializations were avoided. On the other hand, the Eocene Tarsioidea showed progressive tendencies which seem to adumbrate the later development of primates of a definitely pithecoid status.[1] This is shown particularly in the expansion of the brain, the construction of the tympanic region of the skull, and the premolar and molar pattern of the teeth. The available evidence suggests that the higher primates may have had their origin in the Microchoeridae—an extinct family of Old World tarsioids—and such a transformation is actually suggested in the case of the Eocene genus *Caenopithecus*.

The earliest representative of the Anthropoidea which has been found up to the present is *Parapithecus*, from the Oligocene of Egypt. This little animal is known

[1] For an account of the anatomical evidence bearing on the position of the Lemuroidea and Tarsioidea in primate phylogeny, see Le Gros Clark (1934).

only from the mandible and lower dentition, and, scanty though the evidence is, it suggests a direct transition from a tarsioid form to an extremely primitive type of anthropoid ape (Werth, 1918). This interpretation is of particular significance in relation to the view held by some authorities that the catarrhine monkeys (Cercopithecoidea) form a somewhat aberrant group of primates which played no part in the evolutionary development of the anthropoid apes and man. A more advanced form of anthropoid ape is represented by the lower jaw of *Propliopithecus*, also from Oligocene deposits in Egypt, and this fossil leads naturally on to *Pliopithecus* (middle Miocene) and *Prohylobates* (lower Miocene), both of which are remarkably similar to the modern gibbon, *Hylobates* (see O. Abel, 1934). With the discovery of a somewhat specialized gibbon, *Limnopithecus*, and a primitive chimpanzee, *Proconsul*, from Miocene deposits in Kenya, it becomes evident that at the beginning of the Miocene the main groups of the anthropoid apes which exist to-day were already undergoing separately their evolutionary definition. The importance of this fact is related to the general belief (based on comparative anatomical data) that man and the modern anthropoid apes had their origin in a common ancestry, and that the human line of descent became first differentiated at the time when the modern genera of anthropoid apes commenced their own divergent specializations. Hence it is in the palaeontological records of this geological period that the initial appearance of the Hominidae is probably to be sought.

## II. FOSSIL MAN-LIKE APES

Just over one hundred years ago (in 1837) the lower jaw and teeth of a fossil anthropoid ape were found in Miocene deposits in France, and were described by Lartet. Some years later (1856) this distinguished anatomist referred the remains to a new genus, *Dryopithecus*, which, he emphasized, approximated in some respects more closely to man than any of the existing anthropoid apes. Since this discovery, numerous other fragments of the same genus and of allied genera have been recovered from Miocene and Pliocene strata in many parts of the Old World, ranging over western Europe, Egypt, South Africa, India and China.[1] Except for two fragmentary limb-bones, these remains consist entirely of teeth and portions of the upper and lower jaws. Consequently it has proved somewhat difficult to classify them satisfactorily, for it is frequently impossible to determine whether the morphological differences in size and cusp-pattern of the teeth of different specimens are of a specific nature, or whether they are simply an expression of individual or sexual variation. That such a confusion has occurred in some instances is certain, and indeed must be regarded as inevitable, bearing in mind the fragmentary nature of the fossil material and considering also the remarkable variability in the conformation of the teeth shown by the modern anthropoid apes (Remane, 1922). Nevertheless, between twenty and thirty different species of these extinct apes have been recognized. Some of them are of quite a generalized character morphologically.

[1] The most extensive discoveries of dryopithecine apes have been made in geological deposits in the Siwalik Hills of North India. See Pilgrim (1915, 1927). For a general and well-illustrated account of fossil apes, see O. Abel (1931).

Others display structural approximations to the existing genera of apes. For example, the lower Pliocene species *Dryopithecus rhenanus* (*seu germanicus*) shows many similarities to the chimpanzee; *Dryopithecus chinjiensis* (from the middle Miocene levels of the Siwaliks) and *Palaeopithecus* (from the lower Pliocene of the Siwaliks) resemble the gorilla in some of their dental characters; and *Palaeosimia* (from the Siwalik deposits and probably of middle Miocene date) appears to be closely related to the modern orang.

There is good reason, therefore, to suppose that the *Dryopithecus* group and allied genera of Miocene and Pliocene apes were at that time undergoing an active differentiation, leading to the production of a number of separate evolutionary radiations which presumably culminated in the appearance of the modern genera of anthropoid apes. Further, as we have already noted, there is some evidence that this differentiation had already proceeded far even during the Miocene, and that the differentiation of modern genera commenced at least at the beginning of this period. Hopwood (1933), for example, has described a fossil anthropoid from lower Miocene deposits in Kenya, under the name of *Proconsul africanus*, which in its dental anatomy is remarkably similar to the chimpanzee. It is certainly more primitive; indeed, the molar pattern is stated in a recent paper to preserve features reminiscent of a tarsioid ancestry (Gregory *et al.* 1938). Nevertheless, it may be accepted as a primitive representative of the chimpanzee line of evolutionary development, indicating therefore that this line had already in the early Miocene undergone a differentiation from generalized dryopithecine ancestors. If this inference is correct, it has an important bearing on the antiquity of the human phylum, for, according to a general consensus of opinion among comparative anatomists (as already noted), the Hominidae began to be differentiated from the common ancestral group at the time when the modern genera of large anthropoid apes were becoming divergently specialized.

Some species of Miocene apes do, in fact, already show rather remarkable resemblances to man in their dentition. Pilgrim (1927) has emphasized this similarity in the case of *Sivapithecus*, mainly because of the generalized characters shown in the breadth index of the molar and premolar teeth, and the shortness of the symphysial region of the jaw. On the other hand, the canines show some degree of specialization, and according to Gregory & Hellman (1926) this extinct genus of anthropoid apes (to which they also allocate the remains previously designated *Dryopithecus cautleyi*) approximates somewhat more closely to the orang than to the other recent apes or to man.

So far as European fossil apes are concerned, *Dryopithecus rhenanus* has been generally considered to be nearly related to the type from which the Hominidae were directly derived, since some of the molar teeth referable to this species approximate quite closely to those of modern man. There is, however, another species, *Dryopithecus darwini* (= *Griphopithecus suessi* of a previous nomenclature), in which the molar teeth resemble those of man so closely in their general proportions and in the proportionate development of their cusps that they are not easily distinguished from human teeth. Admitting these morphological similarities,

however, the question arises as to whether the Miocene and Pliocene apes of the *Dryopithecus* group all showed any specialized features which would theoretically debar them from consideration in human ancestry. This is certainly not the case with the molar teeth, nor does the conformation of the mandible offer any difficulty. Smith-Woodward (1914) has demonstrated how readily a gradational series can be arranged to illustrate the structural transition of the symphysial region of the jaw

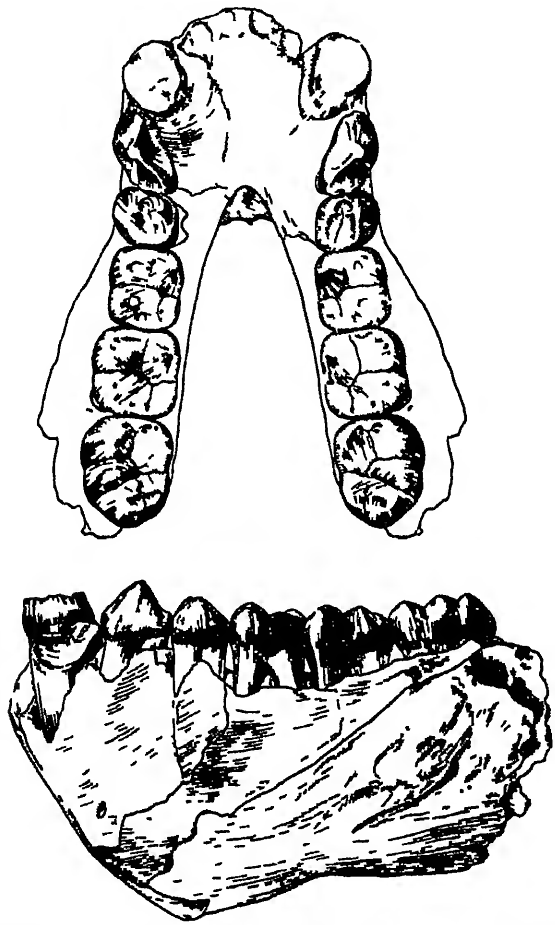

Fig. 1. Composite restoration of the mandible and lower dentition of *Dryopithecus*, made from the parts of three different specimens. This restoration demonstrates the essentially simian character of the *Dryopithecus* dentition. ×9/10 (From Gregory & Hellman, 1926)

from *Dryopithecus* through *Homo heidelbergensis* to modern man. On the other hand, some morphologists have regarded the dryopithecine canines as too highly specialized (already indicating a divergent modification leading to recent apes). It may be noted, however, that the development of the canine in these fossil apes actually showed considerable variation, certainly reaching rather a massive development in some species (e.g. *Dryopithecus fontani*), but remaining relatively weak in others (e.g. *D. pilgrimi*). Further, there is some purely morphological evidence that

the modern human canine has undergone some reduction during its later evolutionary history, so that in ancestral forms it may be expected to show a stronger development.

In recent reports on fossil apes discovered in the Siwalik Hills by the Yale-Cambridge India Expedition of 1935 (Lewis, 1934; Gregory *et al.* 1938) reference is made to two new genera, *Bramapithecus* and *Ramapithecus*, in which the structural approach to man is said to be still more emphatic. This is particularly the case with *Ramapithecus*, a genus known from fragments of upper and lower jaws found in Pliocene deposits. To be precise, their geological horizon is stated to be either the latest middle Siwalik or basal upper Siwalik. The molar teeth closely resemble those of modern man in their breadth, in their low and rounded cusps, and in the absence of a well-marked cingulum. The premolars are bicuspid in a typically human fashion, the canines and incisors (as judged by their alveolar sockets) are quite small, and there is no diastema. Most significant of all is the indication that the dental arch was evenly curved in parabolic form, and not U-shaped as in the modern apes. Indeed, doubt has even been raised as to the propriety of including *Ramapithecus* in the Simiidae rather than in the Hominidae, and the opinion is expressed by Gregory *et al.* that it was "almost at the human threshold", at least in respect of its known anatomical characteristics. However, a comparison with the Pleistocene apes of South Africa (vide infra), of which portions of the skull are available, indicates that such "progressive" characters of the teeth and palate may quite well be associated with cranial characters which leave no doubt regarding their simian status.

It is evident from this short account that the Miocene apes of Europe and Asia may well have included the direct ancestors of man. They showed a great degree of variation in their dental anatomy, and they were very widespread geographically. It seems probable that they spread out from a centre of evolutionary radiation in Asia (represented perhaps by the fossiliferous deposits of the Siwalik Hills), the ancestors of the chimpanzee-gorilla group migrating westwards to the African continent, and the ancestors of the orang migrating eastwards. The wide distribution of the orang in early times is evidenced by a fossil molar tooth (apparently that of the modern type of orang) which has been found as far north as China (Pei, 1935); yet to-day this ape is limited to the Malayan area.

We have noted that the remains of the Miocene apes of the dryopithecine groups which have hitherto been found are practically limited to jaws and teeth. The only exceptions are a femur and the shaft of a humerus, but the specific identification of these limb bones is not quite certain. The humerus was found in middle Miocene deposits in Haute Garonne in association with the lower jaw of *Dryopithecus fontani*, and was described by Lartet in 1856. In size it corresponds to the humerus of a chimpanzee. The femur was discovered in lower Pliocene deposits at Eppelsheim over a hundred years ago. It was referred by Schlosser to *Dryopithecus*, but because of its general resemblance to a gibbon femur this was questioned by Pohlig in 1895, who created a new genus *Paidopithex* to accommodate the specimen. Two years later Dubois (1897) came to a similar conclusion, and invented the generic term *Pliohylobates* to emphasize his opinion. However, a detailed study of the femur by

Pearson & Bell (1917) has shown that it must have belonged to an ape which in point of size was somewhere between the modern orang and chimpanzee. No gibbon of such a stature has ever been discovered, and it is difficult on these grounds to avoid the conclusion that the femur is really that of an ape of the dryopithecine group. It seems reasonable, therefore, to accept Schlosser's opinion that the Eppelsheim femur does belong to *Dryopithecus*, especially as this authority showed that in its general contour it harmonizes quite well with the humerus of *D. fontani* described by Lartet. If this interpretation is correct, it may be inferred from the characters of the femur that *Dryopithecus* was much more adept in erect bipedalism than any of the large anthropoid apes of to-day. Moreover, a comparison of the femur and the humerus suggests (though not conclusively) that the limb proportions of *Dryopithecus* were by no means so highly specialized for arboreal life as they are in modern apes. In other words, they provide evidence that *Dryopithecus* was a "ground-ape", and there are good reasons for supposing that the human line of evolution took its origin from apes which were adapted to a terrestrial life.

The palaeontological evidence which we have briefly reviewed gives strong support to the thesis that the ancestors of the Hominidae are to be sought in some branch of the early Miocene Dryopithecinae. In order to confirm this inference it is essential that we should obtain evidence regarding the cranial characters and the details of the limb structure of these fossil apes. Indeed, it is probably true to say that no greater service could be done to the study of human palaeontology than a comprehensive and intensive geological investigation of deposits in which dryopithecine jaws and teeth have been found, with a view to discovering remains of other parts of the skeleton.

*Extinct man-like apes of South Africa.* In 1924 the famous Taungs skull was found in a limestone crevice in Bechuanaland. The remains (which consist of the facial skeleton with jaws and teeth, a portion of the cranium, and a natural endocranial cast) are those of an immature individual in which the first permanent molar was in the process of eruption. They were described by Dart (1925, 1929) under the name of *Australopithecus africanus*, and have since been studied in considerable detail by W. Abel (1931). It is now generally accepted by comparative anatomists that *Australopithecus* comes within the zoological category of the anthropoid apes. It has been claimed, however, that in certain features it approaches the Hominidae more closely than any other ape so far known, and for this reason it occupies a position of very considerable importance in the study of human evolution. The human characters of the fossil which have been emphasized are stated to be found in the endocranial cast, the dentition, and the palate.

The natural endocranial cast is not quite complete. Nevertheless, it is possible to get some idea of its total volume. According to Dart this amounts to 520 c.c., but it has also been assessed by Keith at 450 c.c., and by Zuckerman at 500 c.c. Zuckerman's studies (1928) of the growth of the brain in anthropoid apes led him to the conclusion that in the chimpanzee the average expansion of the endocranial capacity which occurs after the eruption of the first permanent molar is about 8%. If, therefore, the endocranial capacity of the Taungs skull is accepted as 500 c.c.,

the capacity of the adult individual would probably have been about 540 c.c. This capacity falls well within the range of variation of adult male gorillas (450–655 c.c.). So far, then, as the size of the brain is concerned, *Australopithecus* shows no approximation to a human status. Certain surface features of the endocranial cast have been otherwise interpreted. Particularly is this the case with the lunate sulcus on the occipital lobe. It has been affirmed that the position of this sulcus can be recognized by a furrow on the cast, and that its caudal displacement (as compared with the brains of modern apes) is indicative of a pronounced expansion of the parietal association areas of the cerebral cortex. However, a recent study of the chimpanzee (Le Gros Clark *et al.* 1936) has demonstrated how impossible it is to identify with certainty the lunate sulcus on an endocranial cast. Indeed, it seems probable that the groove which has been taken to represent this sulcus in the Taungs specimen is really formed in relation to the lambdoid suture of the skull.

It must be admitted, in spite of several statements to the contrary, that the endocranial cast of *Australopithecus* does not provide any valid evidence to show that the convolutional pattern of the brain was more human than that of a gorilla. It remains possible, however, that a detailed comparative study of the general contour and proportions of the cast and of its various lobes may yield some more positive information bearing on this problem.

The teeth of *Australopithecus* have been studied in considerable detail by a number of anatomists (apart from Dart who originally described them), but particular attention has been given to their human characters by Broom (1929) and Gregory & Hellman (1939*a*). It has already been mentioned that the skull of *Australopithecus* is that of an immature animal in which the milk dentition is still in place and the first permanent molar is in the process of eruption. The human characters of the dentition which have been emphasized by Broom are: (1) the incisors are implanted vertically and do not show the slight procumbency characteristic of the young gorilla and chimpanzee; (2) the canine is small and of human shape; (3) the first milk molar shows a cusp pattern which closely resembles that of man, and it has an anteroposterior diameter greater than that of the milk canine; (4) the second milk molar, while very similar to that of the gorilla, has less developed and more rounded cusps. Broom's general conclusions (1938*a*) are that *Australopithecus* may be regarded as "a chimpanzee with human teeth".

Gregory & Hellman have concentrated their attention on the characters of the first permanent molar, and they regard the arrangement of cusps and grooves on the crown as intermediate between the *Dryopithecus* pattern and the molar pattern of primitive man.

The shape of the palate of the *Australopithecus* specimen is somewhat shorter and broader than that of immature anthropoid apes, and in this respect, as well as in the contour of the dental arch, it is certainly more human in type.

There can be no reasonable doubt as to the human characters of the milk dentition and the palate of *Australopithecus*. What still remains uncertain is the significance of these characters. It has already been mentioned that the dentition of modern apes shows an unusual range of variation, and it will be necessary to

institute a comparison with a large number of specimens before it can be shown just how far the dentition of *Australopithecus* exceeds the normal range of variation in the gorilla and chimpanzee and approaches that of man. The milk incisors and canines of the gorilla, for example, may on occasion be quite small. On the other hand, it is doubtful whether the milk molars of either the gorilla or the chimpanzee ever approach the human form so closely as do those of *Australopithecus*. One feature which has not received the attention it deserves (but which has been emphasized by Keith (1931)) is the massive character of the first permanent molar. It can only be inferred from this that the whole molar series of the permanent dentition must have been similar in general dimensions to that of a gorilla, and it is hardly to be doubted that such a dentition must have been associated with massive jaws and a large palate. One of the main difficulties in the interpretation of Dart's specimen of *Australopithecus* lies in the attempt to assess the characters of the adult from those of an immature individual. This applies particularly to the brain, the jaws and the teeth. However, since the original discovery of *Australopithecus* in 1924, further remains of adult specimens of similar apes have come to light. We may now refer briefly to this important fossil material.

In a series of communications to *Nature* (1936–8), and more recently in the *Annals of the Transvaal Museum* (1939 *a*), Broom has given brief descriptions of these remains, and has created two new genera to accommodate them—*Plesianthropus* and *Paranthropus*, the former having been first regarded as a new species of *Australopithecus*. It is not quite clear why he makes a generic distinction from *Australopithecus* in either case, for the similarities with the latter seem to be quite close. The remains of *Plesianthropus* were found in a limestone quarry at Sterkfontein. They are represented by a portion of an adult skull, the maxilla and upper teeth, and a natural endocranial cast. In regard to the teeth, the canine is said to be hardly larger than that of man, and to be worn down by attrition to the same level as the first premolar and the lateral incisor. The premolars are typically bicuspid as in man, the first permanent molar is moderately large and shows a typical dryopithecine cusp-pattern, and the second molar is "exceptionally large". The palate is relatively broad and the dental arch rounded. The endocranial cast is estimated to indicate a cranial capacity of about 440 c.c.

The skull of *Paranthropus* (consisting of a considerable part of the left side of the basal portion of the cranium and the right side of the mandible) was found at Kromdraai two miles from Sterkfontein in an outcrop of bone breccia near the top of a hill. The facial skeleton is reported to be shorter and flatter than that of a gorilla, though in its general size it approximates to this anthropoid ape. The canine and incisors are relatively small, the premolars have rounded crowns without the prominent and sharply-defined cusps found in modern apes, and the palate is relatively short and broad. From the fragments of the endocranial cast Broom estimates the cranial capacity to be as high as 600 c.c. However, he does not indicate how he arrived at such a figure: indeed, it seems from the available evidence that it is hardly possible in this case to make an estimate which is even approximately accurate.

In their account of the fossil fragments of *Plesianthropus* and *Paranthropus*, Gregory & Hellman (1939*a*, *b*) confirm Broom's general conclusions regarding the human characters of the palate and dentition, and also call attention to the way in which the occlusal surfaces of the molars have been worn flat, indicating a masticatory movement similar to that of man.[1]

In summarizing the characters of these extinct apes of South Africa it must be emphasized that detailed descriptions of all the material are not yet available, and that, apart from a brief inspection of the original fragments of *Australopithecus* some years ago, the present author has had to rely for his own observations on casts, photographs and drawings. Moreover, some of the descriptions which are available (particularly in the case of *Plesianthropus* and *Paranthropus*) are rather meagre, and

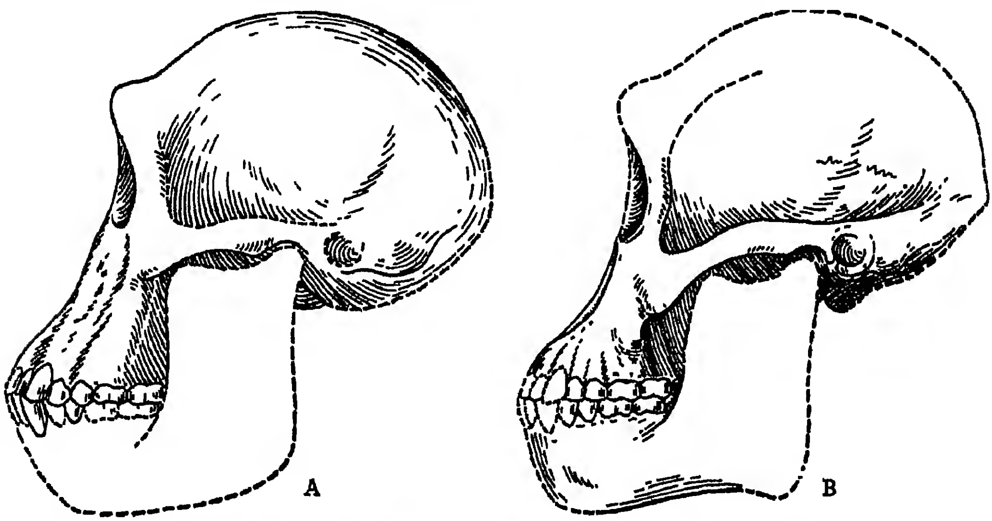


Fig. 2. Provisional restorations of the skulls of (A) *Plesianthropus*, and (B) *Paranthropus*. × 1/2 *circa*. (From Gregoiy & Hellman, 1939*a*.)

it seems probable that certain of the conclusions which have already been drawn concerning the human characters of these fossils have been based on preliminary observations without an adequate comparative study, and without taking into account the variability shown in the teeth and skulls of modern anthropoid apes. We may accept as an established fact that the teeth and palate are in several respects certainly more human than any of the living anthropoid apes. On the other hand, there is no room for doubt that these fossil genera are really apes and not primitive types of humanity. This is definitely shown to be the case by the size and contour of the skull and endocranial cast, by the massive nature of the jaws, and by the nasal and orbital

[1] In a recent communication to *Nature* (19 Nov. 1938, p. 897) Broom has figured and very briefly described the lower end of a humerus which he ascribes to *Paranthropus*, and the lower end of a femur ascribed to *Plesianthropus*. Both these fragments are stated to be "nearly human". However, in the absence of adequate geological evidence, there must be considerable doubt whether such associations are correct, particularly as the fragments were picked out of a mass of skeletal remains of diverse character. In any case, it is necessary to await a careful and critical comparative study of these limb bones before their true significance can be assessed.

regions of the skull. Certain features of the endocranial cast, and other characters such as the plane of the foramen magnum and the conformation of the tympanic region, have been held to show a "progressive" trend, approximating in some degree to human level, but it is probable that in none of these features will such a conclusion withstand a critical analysis based on an adequate comparative study.

What, then, is the real significance of the human characters of the palate and dentition in these specimens? There can be no justification for supposing that the South African fossil apes bear a direct ancestral relationship to *Homo*, for the geological and faunal evidence shows that they almost certainly lived in Pleistocene times[1] when early representatives of the Hominidae were already in existence in other parts of the world. There are two other alternative interpretations. The fossil apes may represent a survival into Pleistocene times of an anthropoid stock which at an earlier date gave rise to man, or they may represent a separate line of evolution which was characterized by an independent and parallel development of certain features of the dentition and palate similar to those of man, but having no close relation to the latter. It must be frankly admitted that, with the evidence at present available, it is not possible to decide on this issue. W. Abel (1931) has drawn attention to certain features of the teeth of *Australopithecus* which he believes to be too highly specialized to provide a basis for the evolutionary development of the human type of dentition, and such evidence must clearly be taken into consideration. From other points of view, however, these fossils have an important bearing on the evolutionary origin of the human phylum. There can be no reasonable doubt that they were derived from a dryopithecine ancestry of Miocene date, and they thus demonstrate that the dryopithecine stock was endowed with a potentiality for evolutionary development in the direction of the Hominidae at least in so far as the teeth and palate are concerned. It has also been emphasized (by Dart (1929) and by Gregory & Hellman (1939*a*)) that the fossil apes of South Africa show many resemblances to the orang as well as to the gorilla and chimpanzee. This combination of generic characters suggests that they were ultimately derived from extremely generalized dryopithecine ancestors at an evolutionary phase in which the three modern genera of apes had not become differentiated from a common ancestral stock. If the South African fossil apes, therefore, are regarded as descendants of the ancestral stock from which man arose, it must be accepted also that the evolutionary differentiation of man is to be referred back to a correspondingly early phase in the evolution of the higher primates.

## III. FOSSIL APE-LIKE MEN

In 1891 the remains of an extremely primitive type of man, *Pithecanthropus*, were discovered by Dubois at Trinil in Java. As is now well known, the remains consisted of a skull-cap, a femur and three teeth. The skull-cap showed some

[1] The precise date of these fossils remains uncertain. In his latest estimate (which, however, is of a provisional nature) Broom supposes that *Plesianthropus* and *Paranthropus* date from the middle Pleistocene, and *Australopithecus* from the lower Pleistocene or upper Pliocene. However, there seems to be no satisfactory evidence for a date so early as the latter.

features which were so remarkably simian in appearance that anatomists were at first in considerable disagreement regarding its significance. Some accepted it as human, others believed it to be part of a gigantic gibbon, while still others looked upon it as a real "missing link". For thirty-eight years no other fossil remains similar to *Pithecanthropus* were known. Consequently, the fragments of the type specimen have been subjected to exhaustive study by anthropologists all over the world. In spite of one or two dissentient opinions, there is now almost unanimous agreement that *Pithecanthropus* comes within the category of the Hominidae. Such a conclusion is established with certainty by the features of the endocranial cast (as first emphasized by Elliot-Smith). The cranial capacity is estimated to be between 900 and 1000 c.c. Although this is low compared with the average capacity in modern man (about 1350 c.c.), it comes within the extreme limits of its variation, while the highest cranial capacity hitherto recorded for an ape is 655 c.c. (in a male gorilla). Apart from its size, however, the endocranial cast of *Pithecanthropus* shows a number of primitive features which serve to distinguish it from that of *Homo sapiens*, such as the general flattened shape and angular contour of the cerebral hemispheres, the narrow frontal lobes, and the attenuated temporal lobes. So far as the fissural pattern of the brain can be inferred from the cast there is evidence that this also was in some respects more simian than in many modern human brains.[1]

The calvarium of *Pithecanthropus* is characterized by a forehead region which is narrow, flattened and retreating, a pronounced supraorbital torus, a projecting occiput, and an extensive area for the attachment of the nuchal musculature.

The femur, in contrast to the skull-cap, is in all respects similar to that of modern man. This statement is deliberately made in spite of the fact that Dubois himself (who is a supporter of the giant gibbon hypothesis) has sought on more than one occasion to prove that it possesses ape-like characters. Such an interpretation, however, has not been confirmed by other anatomists. In a recent comparative study of the long axes of the femur in man and the anthropoid apes, Walmsley (1933) concludes that in regard to the position of the load line, that is to say, in "the position of the bone as a functioning structure, as has often been pointed out of its other characters, the Trinil femur is fully modern". He further notes that, although in the relative length of the condyles and the convexity of the popliteal surface (on which Dubois lays such stress) it "diverges towards the gibbon", it does not do so "more than some modern femora do". The modern characters of the femur have inevitably raised the question whether it can legitimately be associated with such a primitive type of skull, and this doubt is reinforced by the fact that the thigh bone was discovered a considerable distance from the cranial fragment. However, they were both retrieved from the same geological stratum (the Trinil horizon, as it is now called), and, as we shall see, a similar combination of skeletal characters has since been found in other fossil remains in China.

The teeth discovered by Dubois and related by him to the skull-cap and femur are considerably worn and difficult of interpretation. Probably they should now be

[1] The endocranial cast of *Pithecanthropus* has in recent years been carefully studied by Kappers (1929). See also Kappers & Bouman (1939).

left out of consideration, for it is regarded as likely by some authorities that they are really the teeth of an orang. It may be noted that Dubois (1924) found among the fossil material which he collected a tiny fragment of a mandible, which he also attributes to *Pithecanthropus*. However, this piece of bone came from deposits at Kedung Brubus, about 24 miles from the locus of the Trinil calvarium, so that

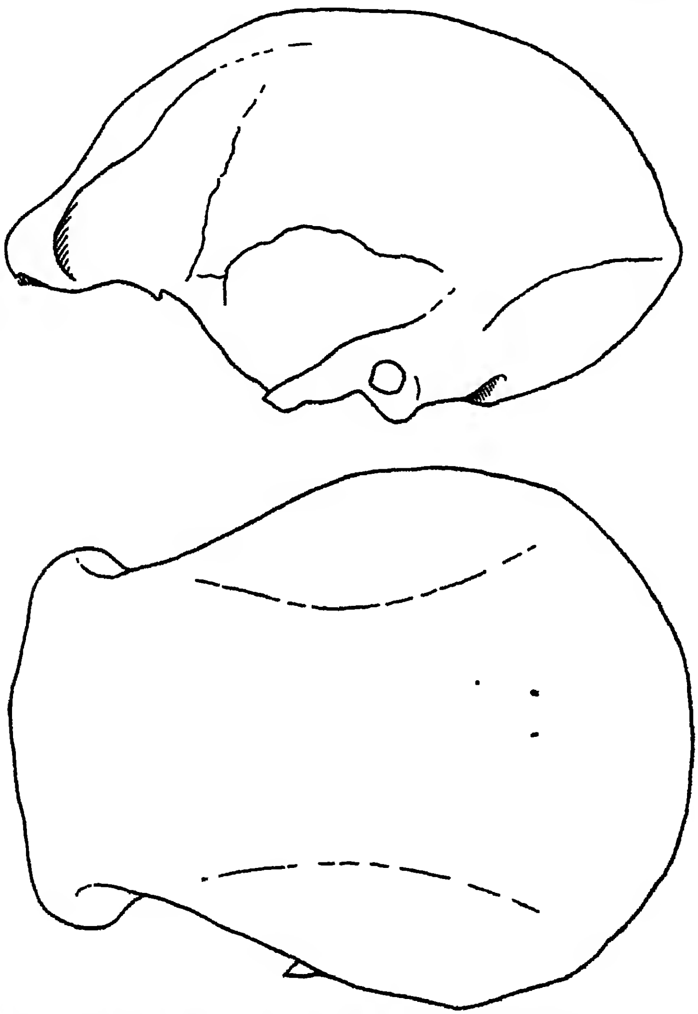

Fig. 3. Lateral and dorsal views of the skull of *Pithecanthropus* described by v. Koenigswald. × 1/2. (Tracings of photographs by v. Koenigswald, 1938.)

its identification is (to say the least) by no means certain. In any case, it is too small to give any very useful information regarding the character of the mandible as a whole.

As we have already noted, for many years after Dubois' initial discoveries no further remains of *Pithecanthropus* were found, although several geological expeditions were organized and dispatched to Java to search for them. This long

period of fruitless excavation has been broken quite recently. In August 1937 an adult skull was discovered in the deepest layers of the Trinil horizon at Bapang in Java, and this material was described in detail by v. Koenigswald (1938). It shows remarkable similarities to Dubois' *Pithecanthropus*, as, for instance, in the contours of the vault of the skull, the general platycephaly, the extreme flattening of the frontal region, the postorbital constriction, and the heavy supraorbital ridges; indeed, it cannot be doubted that it belongs to the same genus (and probably the same species) of primitive man.[1] This new specimen is of particular importance because a part of the base of the skull has been preserved. It shows that the glenoid articular cavity, and therefore the temporo-mandibular joint, was precisely similar to that of modern man. It may be inferred from this that the dentition was essentially human in its general characters. On the other hand, the mastoid process appears to have been undeveloped, a character in which some approach is made to the anthropoid apes.

The cranial capacity of the new *Pithecanthropus* skull is estimated by v. Koenigswald to be no more than 750 c.c., a surprisingly low volume even when compared with the estimated 900–1000 c.c. of the original specimen. It is suggested that the contrast may be related to a sexual difference. The endocranial cast has been studied by Bouman (1938), and compared with that of Dubois' specimen by Kappers & Bouman (1939). In these studies it is clear that the general form of the endocranial cast and the appearance of the convolutional pattern (so far as it can be inferred) are astonishingly alike in both cases.

Another fossil fragment of considerable importance which can be with confidence assigned to *Pithecanthropus* is the lower jaw found at the end of 1936 in the neighbourhood of Sangiran (Solo) in Java (v. Koenigswald, 1937). It comprises the horizontal ramus of the mandible with the second premolar and the three molar teeth. It shows that the dental arch was typically human in its general conformation; the first premolar (as seen from its empty socket) had but one root; the molar teeth have a general resemblance to those of Neanderthal man, and their length-breadth index is quite human. The chin region was retreating, but there was no indication of a "simian shelf". The whole bone was evidently a massive structure, a good deal stronger than in modern man, and bearing a general resemblance to the well-known Mauer jaw (*Homo heidelbergensis*). It is also noted by v. Koenigswald that it harmonizes quite well with the small mandibular fragment found by Dubois at Kedung Brubus many years previously.

Perhaps the most remarkable discovery in Java during the last few years is the skull of an infant found in February 1936 at Modjokerto (near Soembertengah). It was derived from a geological stratum containing what is termed the *Djetis* fauna. According to v. Koenigswald (1936), in numerous places in middle and east Java the strata containing the Trinil fauna are found above those associated with the

[1] It should be observed that the skull was crushed into a number of fragments on its discovery, and these had to be carefully pieced together. v. Koenigswald has been criticized from some quarters for the manner in which he has reconstructed the fossil, but there is actually no reason at all for doubting the essential accuracy of his careful work.

*Djetis* fauna; in other words, the latter are the more ancient. Since it is now generally accepted that the Trinil horizon is of middle Pleistocene date, the Modjokerto skull must be at least early Pleistocene. Such a conclusion is borne out by certain elements of the *Djetis* fauna (e.g. *Epimachairodus*, *Nestoritherium*, and *Leptobos*) which are referable to the latest Pliocene or the earliest Pleistocene.

The Modjokerto skull is clearly that of a very young individual. It has a maximum length of 138 mm. and a maximum breadth of about 115 mm. From the appearance of the bregmatic region the age of the infant was certainly more than two years. On the other hand, the parietal bones are extremely thin (about 3 mm.) and the

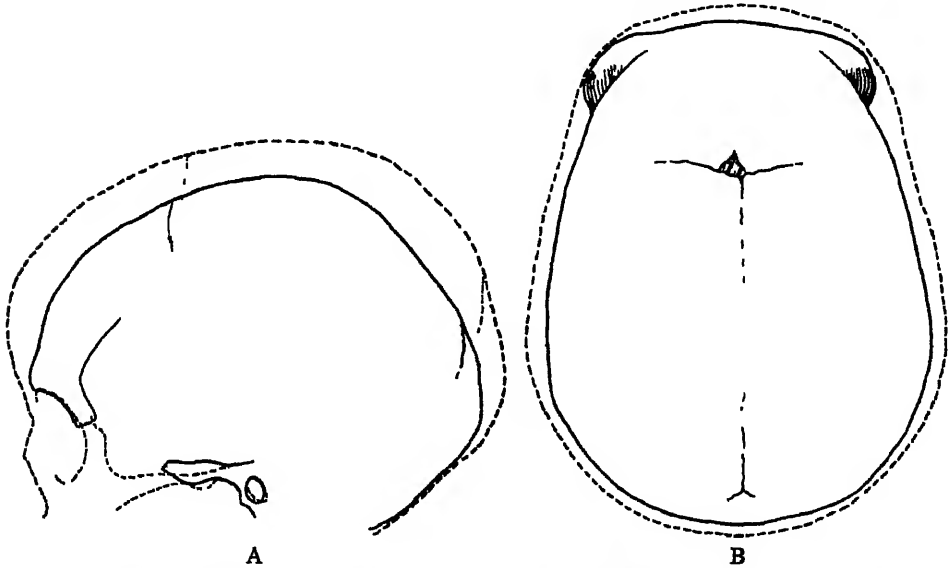

Fig. 4. Lateral and dorsal views of the Modjokerto skull, superimposed on the outline of the skull of a European child of three years. × 1/2 *circa*.

tympanic region of the skull is well ossified. Moreover, the supraorbital ridges are already becoming prominent, the forehead shows a relatively retreating shape, and there is an incipient postorbital constriction. Taking into account all the features available, it seems probable that the Modjokerto infant had reached a stage of development equivalent to a modern child of six years. The glenoid cavity shows that the temporo-mandibular joint is typically human. The cranial capacity is estimated to be about 700 c.c., and by reference to data concerning the brain growth of modern man and the anthropoid apes it may be inferred that this would not have expanded beyond 1000 c.c. in the adult. There is no reason to doubt, indeed, that the mature form of the Modjokerto baby must have been quite similar to *Pithecanthropus*, as the latter is known from fully developed skulls.

It may be observed that even if the estimate of six years for the age of the skull is open to question, this does not affect the unusual importance of the specimen from the anatomical point of view. If the individual were older than this, then the unusually small volume of the brain would be further emphasized; if it were

younger than six, the remarkable development of the supraorbital eminences, the retreating character of the forehead, and the advanced degree of ossification of the tympanic region would all assume a greater significance. That the skull of a baby *Pithecanthropus* should *appear* less obviously primitive and simian than the adult form is of course to be anticipated from reference to comparative data, for even in the immature skull of anthropoid apes the brain case is more rounded and relatively larger in relation to the facial skeleton, the brow ridges and muscular eminences are much less developed, and the jaws are not so prominent—in other words, the young ape skull is more like that of man than the mature structure is. These facts, indeed, provide the basis for the conception of paedomorphosis as an evolutionary phenomenon.

We may now turn our attention to recent discoveries relating to early Pleistocene man at Choukoutien, near Pekin. These remains have been assigned to a new genus *Sinanthropus*, and the first recognition of their importance is due to the study of a single molar tooth by Black in 1927. The story of the research which led to the extensive and systematic excavation of the Choukoutien site has been told on several occasions,[1] and is now well known to palaeontologists. Black's classical description (1929*a*, 1930*a*) of the first skull to be found (in Locus E of the Choukoutien deposits) gives a general idea of the main characters of the Pekin man. The skull is very similar to that of *Pithecanthropus*. The forehead region is narrow, flattened and retreating, the supraorbital ridges are very powerfully developed, the mastoid process is poorly developed, the markings for the nuchal musculature are very prominent, the whole skull is markedly platycephalic, and the bones are extremely thick. The cranial capacity of this skull was estimated by Black to be about 960 c.c.

Since the discovery of the first skull, many other remains have come to light. A second skull was found in 1929 at Locus D (Black, 1930*a*), a third in 1929 at Locus E (Black, 1931), and three adult skulls in 1936 at Locus L (Weidenreich, 1937*a*). In addition, numerous fragmentary remains of other crania have come to light, as well as many teeth and pieces of jaw. Altogether, the material which has now accumulated represents more than 30 different individuals of different ages, and it may be regarded as fairly representative of the early Pleistocene population of this district.[2]

A study of all the available material of *Sinanthropus* has emphasized the great variability shown by this extinct type of man. The cranial capacity of adult skulls, for example, ranges from 850 to 1220 c.c. according to Weidenreich. In other words, at its upper limit it comes well within the range of variation of modern man. The forehead region may even be fairly well rounded, though this character tends to be obscured by the prominence of the supraorbital ridges. It is possible, of course, that these variations may be related to sexual differences, but of this there is no certainty. The jaws were clearly rather massive and showed a high degree of prog-

[1] See, for example, Elliot-Smith (1931*a*, *b*) and Keith (1931).

[2] The geological and faunal evidence bearing on the age of the Choukoutien deposits has been the subject of numerous papers published during the past few years in *Palaeontologia Sinica* and the *Bulletins of the Geological Society of China*. See particularly Pei (1931).

nathism. The chin is retreating (as in Neanderthal skulls), though an incipient mental eminence is usually evident. According to Weidenreich, in some respects the symphysial region (particularly on its inner aspect) is more advanced morphologically than it is in Neanderthal man. On the other hand, the frequent presence of multiple mental foramina may be regarded as a simian trait.

In a recent publication Weidenreich (1937*a*) has described the dentition in considerable detail. His description is based on the study of 147 teeth from the main deposit at Choukoutien, probably representing 32 individuals of different ages, and is well illustrated by drawings, photographs and radiographs. The most

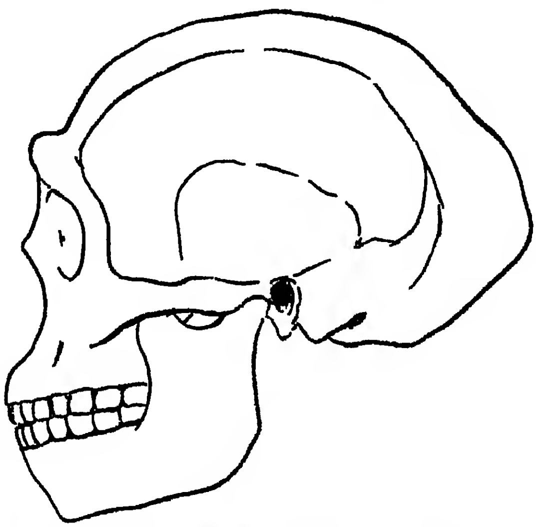

Fig. 5. The skull of *Sinanthropus*, as restored by Weidenreich (1937*a*). × 2/5 *circa*.

striking feature of the teeth is their size, and the fact that this seems to show a pronounced sexual difference is in itself a primitive feature of some significance. The upper incisors are large and spatulate. The lower incisors, on the other hand, are small and closely resemble those of modern man. The molar teeth are characterized by the complexity of the cusp pattern which, however, in its general features conforms to that of *Homo*. The lower premolars are somewhat distinctive, for in addition to their size they show some resemblances to the anthropoid apes in the proportions of the crown and in the arrangement of the cusps. The canines are significantly massive, but it is doubtful whether some of the simian characters which have been ascribed to them really exist. Weidenreich has emphasized the pointed shape of their crowns, and also states that they projected well beyond the level of the adjacent teeth. However, he seems to have arrived at this conclusion by the comparison of isolated teeth, and by assuming that the basal margin of the enamel provides an accurate base-line for estimating the level of the biting surface. Such an assumption is not correct, and indeed this is immediately seen to be the case by reference to fig. 239 in his monograph, which shows the "restored" dentition of *Sinanthropus* compared with the dentition of modern man. In *Sinan*-

*thropus* the canine is made to appear markedly projecting, but if the modern human teeth had been orientated in the same way (i.e. with the basal margins of the enamel occupying the same horizontal plane), the canine tooth here would also appear projecting. Reference to the other figures given by Weidenreich (e.g. fig. 345) demonstrates clearly enough that the canine of *Sinanthropus* is just as "brachydont" as in modern man, and, indeed, the nature of the wear of all the teeth, as well as the anatomical characters of the temporo-mandibular joint, shows that the canines could not have projected as they do in the large anthropoid apes (Fig. 7).[1]

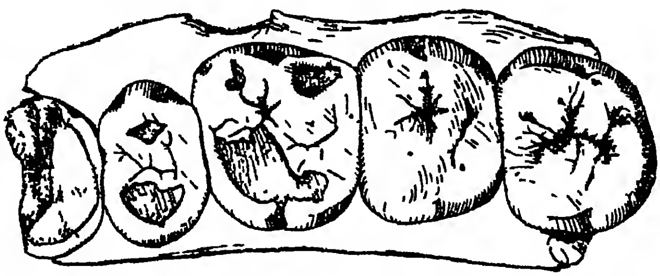

Fig. 6. The upper molar and premolar teeth of *Sinanthropus*—skull 2, Locus L. ×2. (From Weidenreich, 1937*b*.)

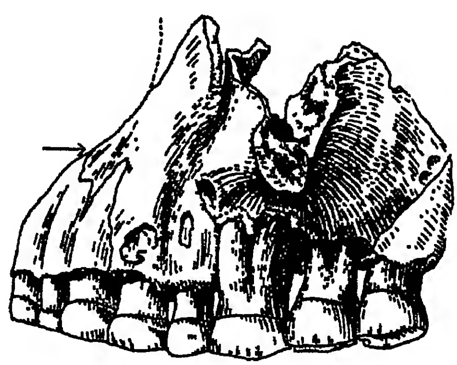

Fig. 7. The left upper jaw and teeth of *Sinanthropus* (Locus O I). The first incisor and the canine have been restored from original *Sinanthropus* specimens. This illustration emphasizes the hominid character of the dentition of *Sinanthropus*. Nat. size. (From Weidenreich, 1937*b*.)

The dental arch is typically human in its conformation, making an even curve, and the canines are implanted alongside the incisors with no break in the series. The enlarged pulp cavities of the molars (as seen radiographically) somewhat approach the taurodont condition of Neanderthal man, in contrast to their appearance either in recent man or in the anthropoid apes. The roots of the molars show

[1] I must admit that I was previously misled by the published description of the dentition of *Sinanthropus*, for in a recent publication (1939) I stated that "the teeth are in many respects astonishingly simian". A more detailed study of the evidence suggests that this statement is rather too emphatic. Black (1929*a*) had arrived at the conclusion from a study of the mandible of *Sinanthropus* that the root of the canine in the adult was "but slightly longer and more massive than those of the premolar".

essentially hominid relations to the dental canal. Indeed, the whole appearance of the dentition can hardly fail to demonstrate its general hominid characters (as Black first recognized). It is surprising, therefore, to find Weidenreich saying that, if the characters of the teeth are employed as a criterion of classification, "there is no other choice but to range *Sinanthropus* within the general group of anthropoids". In spite of certain simian features such as are presented by the large size of the teeth, the characters of the lower premolars and some details of the molar cusp pattern, this statement can only be regarded as an unfortunate and misleading hyperbole.[1] Incidentally, it may be noted that the jaw and teeth of *Pithecanthropus* from Java which have been recently described by v. Koenigswald appear to resemble very closely those of *Sinanthropus*.

The endocranial cast of *Sinanthropus* was first accurately described by Black (1933). Apart from his tentative suggestion that the "anterior insular region was most probably exposed",[2] he drew the conclusion that "the convolutional pattern of the cerebrum so far as it may be determined from the cast with any degree of certainty is such as might be expected to obtain in any primitive human brain". The endocranial cast has also been described by Shellshear & Elliot-Smith (1934) and by Weidenreich (1936). In the former report certain speculations regarding the fissural pattern of the brain are offered, and particular reference is made to the assumed presence of a sulcus lunatus of simian type. However, there is little doubt that the groove which has been taken to represent a sulcus lunatus is nothing more than the impression of a ridge of bone raised up alongside the lambdoid suture, for such a groove is quite commonly to be seen in endocranial casts of modern human skulls as well as in those of anthropoid apes.

We may now refer to some of the limb bones of *Sinanthropus* which have been found in the Choukoutien deposits. In 1932 Black described a clavicle, an os lunatum, and what appears to be the terminal phalanx of the hallux. In 1935 Weidenreich described a small fragment of the shaft of a humerus, and more recently (1938) he has recorded the discovery of portions of the shaft of two femora and the greater part of the shaft of a humerus. The striking feature about all these bones is their remarkable similarity to those of modern man. Indeed, it is doubtful whether they show any distinguishing characters at all. The clavicle and humerus indicate an upper limb skeleton which is quite typically human, and the muscular markings on the humerus give evidence of well-developed pectoral and deltoid muscles. The femoral fragments show no simian features. The shaft of the femur was as straight as it is in modern man, and must have been associated with a completely erect posture. The total length of the complete femur is estimated from one specimen to have been 400 mm., corresponding to a body stature of about five feet. Also, so far as can be judged with the available material, the proportions of the

[1] It is somewhat remarkable to find Davidson Black remarking on the contrasting association of hominid teeth with a mandible showing many simian features, while Weidenreich comments on the association of simian teeth with a hominid jaw. Both authorities make these references to the jaws and dentition of *Sinanthropus*!

[2] Some doubt is thrown on the evidence for this inference by the observation that even in a gorilla's brain the insula may be completely submerged (Le Gros Clark, 1927).

upper and lower limbs in *Sinanthropus* were probably the same as they are in *Homo sapiens*.

These observations on the limb structure of *Sinanthropus* are of very considerable importance in the study of human phylogeny. They demonstrate that, at least at the very beginning of the Pleistocene, even though the skull, brain and dentition retained many primitive features, the limbs had already attained their final shape and proportions. This can only mean that the point of divergence of the line leading to man from that leading to the modern anthropoid apes must have been correspondingly remote. In other words, it suggests that the relationship between man and the modern anthropoid apes (particularly the gorilla and chimpanzee) is probably not quite so close as some anthropologists have supposed.

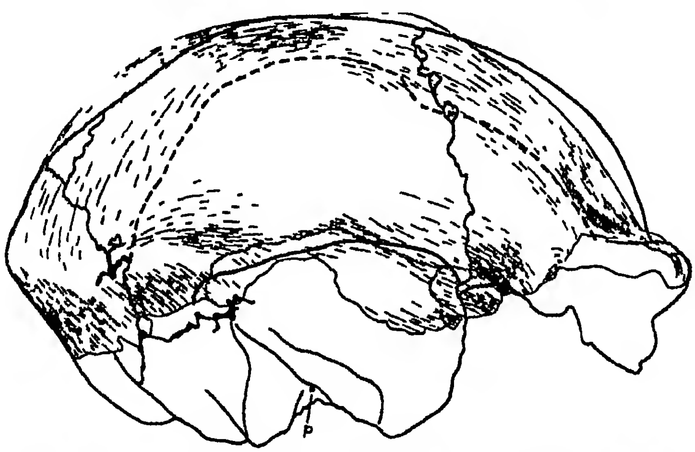


Fig. 8. The contours of the skull of *Pithecanthropus* (shaded) and *Sinanthropus* superimposed on the glabella-inion line. The skull contour of *Sinanthropus* is slightly reduced relatively to conform to the length of the *Pithecanthropus* skull. Abbreviation: *p*, porion. (From Weidenreich, 1937*c*.)

From the account which has been given of the Javanese and Chinese fossils it will be readily apparent that they are very similar indeed in their anatomical characters. The general shape of the cranium, the development of the supraorbital ridges, the range of variation of the cranial capacity, the anatomy of the jaws and teeth, and the strange contrast between an archaic type of skull and a completely modern limb skeleton, are in both cases almost identical. Indeed, it has now become apparent that the two series of fossils represent different geographical groups of the same genus. On the whole, the Chinese fossils suggest a slightly higher grade of development, since the frontal region of the skull is somewhat better developed. For this reason it may be desirable to regard them as belonging to a different species, perhaps under the name *Pithecanthropus pekinesis*, but it is certainly unreasonable to demand a generic distinction.[1] In spite of this, some authorities still persist in separating *P. erectus* of Java sharply from Pekin man, but it is curious to note that

[1] The suggestion of a separate genus, *Sinanthropus*, was no doubt justified when the discoveries were first made. Now that so much material has accumulated to demonstrate the great range of individual variability, there can be no excuse for retaining the distinction.

different arguments are put forward to support such a conception. Dubois holds to his contention that *P. erectus* was a giant gibbon, having no relation to man. On the other hand, he has for some time maintained that the remains found at Choukoutien are those of *Homo sapiens*. He thus finds himself now in the position of having to make this remarkable taxonomic distinction between two groups of fossil material which are almost indistinguishable anatomically! Weidenreich (1937*c*) comes to a very different conclusion. For him *Sinanthropus* is more primitive than *Pithecanthropus*, in spite of the fact that the frontal region of the brain is better developed in the former than in the latter. It must be frankly admitted that neither of these opinions is in accord with the actual facts. Indeed, that two well-known anthropologists can arrive at such divergent conclusions from a study of the same material may well suggest to the general biologist either that the science of physical anthropology is still in a very embryonic stage of development, relying for its interpretations largely on speculation, or that anthropologists allow their judgment to be unduly affected by influences other than those of objective analysis.

From the preceding account of the *Pithecanthropus* group it is evident that at the beginning of the Pleistocene an early type of man inhabited eastern Asia, extending from China down to Java, and characterized by a number of primitive features such as a low brain capacity, retreating forehead, massive supraorbital ridges, prominent jaws, and large teeth. It is not known for certain whether they extended their geographical range farther than this. In 1935 fragments of a human skull were found by Kohl-Larsen in a sandbank by the side of Lake Nyasa. The antiquity of this fossil is doubtful, but according to Weinert's reconstruction the skull approximated in its general features to the *Pithecanthropus* group, and its cranial capacity is estimated at about 1100 c.c. (Weinert, 1937). However, the skull was considerably damaged and it is difficult to accept with assurance the conclusions which have been based on a reconstruction.

The evolutionary origin of the *Pithecanthropus* group remains entirely conjectural. It may be presumed that it was ultimately derived from a dryopithecine ancestry, but there is still a very conspicuous gap here in the palaeontological record.

## IV. NEANDERTHAL MAN

In 1856 the type specimens of Neanderthal man were discovered in a cave of the Neander valley in Germany. Since this date other remains of a similar kind have come to light in many parts of Europe and the Near East, and in his book on the skeletal remains of early man, published in 1930, Hrdlička listed eighteen sites where Neanderthal man has been found. Still more recently, our knowledge of this extinct type has been further extended by other discoveries; of these special attention may be drawn to an exceptionally well-preserved skull found in February 1939 at San Felice Circeo in Southern Italy (Blanc, 1939), a portion of a skull from Saccopastore (Breuil & Blanc, 1935), the skull and parts of the skeleton of an eight-year-old child found during the summer of 1938 in the caves of Teshik-Tash in Southern Usbekistan (detailed report not yet published), and a number of skulls

and limb bones from Mt Carmel in Palestine (a report of which has been published in a recent book by T. D. McCown & A. Keith). All this accumulation of fossil material has served to emphasize the relative homogeneity of the Neanderthal group, to demonstrate its distinction from *Homo sapeins* (see Morant, 1927), and to show its wide geographical range. As regards the antiquity of Neanderthal man, it is now generally recognized that in Europe he was contemporaneous with the Mousterian phase of Palaeolithic culture, and lived during the advance of the Würm glaciation.

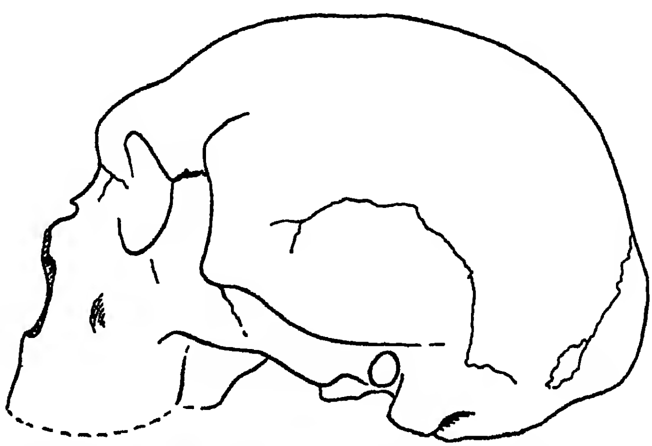

Fig. 9. Lateral view of the Neanderthal skull from San Felice Circeo. (From Blanc, 1939.

While it is not possible to summarize here even briefly the results of the study of all the skeletal remains of Neanderthal man, it is of some importance to draw attention to certain variations which they show. The main skeletal characters are well known—the heavy supraorbital torus stretching in continuity across both orbits, the platycephaly, the receding forehead, the projecting occiput with its massive ridges for the nuchal musculature, the large orbits, prominent jaws, and the absence of a chin eminence, the capacious palate and large teeth, and less obvious details such as the undeveloped mastoid process, the convexity of the facial surface of the maxilla, and the backward disposition of the foramen magnum. In these features Neanderthal man resembles the earlier men of the *Pithecanthropus* group. A rather striking contrast is shown, however, in the size of the brain, for the cranial capacity is known to range from about 1200 to 1625 c.c. Indeed, the average capacity (about 1450 c.c.) is actually higher than that of modern man (about 1350 c.c.).

In the classical examples of Neanderthal man which have been described (e.g. the remains found at Chapelle-aux-Saints, Boule, 1913), the skeleton shows the development of a number of somewhat specialized characters, and it has therefore been inferred that the Neanderthal type could hardly have given rise to *Homo sapiens* in whom these specializations have been avoided, but that it represents an aberrant and abortive side-line in the evolutionary history of the human family. The specializations to which reference is made include an exaggerated development of

the supraorbital torus, a peculiar modification of the molar teeth which has been termed "taurodontism",[1] and changes affecting the limb bones, which are clumsily built with pronounced curvatures of the shaft and enlarged extremities. Such distinctive features are characteristic of the Neanderthal types which lived during the later Mousterian epoch, that is, during the cold period associated with the onset of the Würm glaciation, and the skeletal remains of these types are commonly found in deposits which contain the remains of mammals such as the mammoth (*Elephas primigenius*) and the woolly rhinoceros (*Rhinoceros tichorhinus*). There

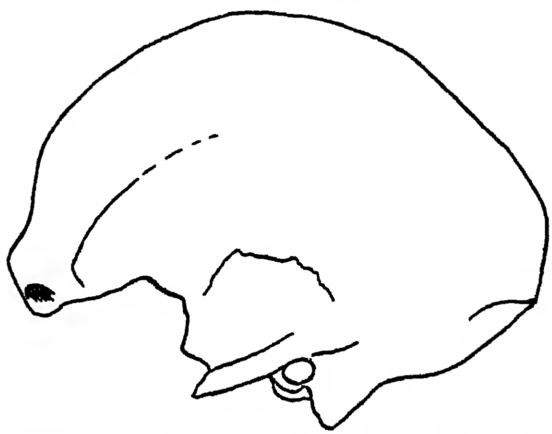

Fig. 10. Lateral view of the Ehringsdorf skull. × 1/3 *circa*.

seems little doubt that these types ultimately became extinct, for no skeletal remains of *later* date have been found which mark a transition to *Homo sapiens*. Moreover, it is well recognized that there is rather an abrupt hiatus in the cultural sequence between the Mousterian phase of the palaeolithic period and the immediately succeeding phases.

Still more significant is the fact that the remains of Neanderthal man dating from the *earlier* part of the Mousterian epoch are often less "Neanderthaloid" in their skeletal characters and, indeed, approximate more closely to *Homo sapiens* than the classical type of later date. In this respect the discoveries at Krapina in Croatia are of special interest. Here the skeletal remains of at least twenty individuals have been unearthed, and they show a considerable degree of variation (Gorganović-Kramberger, 1906). While the characteristic development of the supraorbital ridges seems to be constant, in some of the skeletons features of a progressive nature are evident. For example, in some instances the forehead region of the skull is relatively well developed, the symphysis of the mandible shows the incipient development of a chin eminence, the teeth are very similar to those of modern man, and, although some of the molars show taurodont characters, others lack this specialization. Lastly, the limb bones are slender and straight in comparison with the apparently retro-

[1] The significance of taurodontism as a specialized feature peculiar to Neanderthal man has been to some extent discounted by the observation that the same feature may occasionally be found in the molar teeth of modern man (see Middleton Shaw, 1928).

gressive characters of the more extreme types of Neanderthal man. The Krapina deposits, it should be noted, were evidently laid down during a fairly warm interglacial period, for while the remains of *Rhinoceros merckii* are quite frequent, there is complete absence of *Rhinoceros tichorhinus* and of the mammoth. It appears, therefore, that the Krapina people date at least from the earlier part of the Mousterian epoch.

Similar conclusions may be drawn from other palaeontological evidence, as shown, for example, by the Galilee skull (Keith, 1927), and the Ehringsdorf skull (Weidenreich, 1928). The latter is particularly important, for the geological and cultural evidence indicates that it is either of very early Mousterian date, or, more probably, definitely pre-Mousterian. The skull, which is that of a young adult, is lofty, the forehead region is vertical and well arched as in modern man, the mastoid process is quite strongly developed, the supraorbital ridges are pronounced but not so massive as in typical Neanderthal skulls, and the cranial capacity is estimated at 1450 c.c.

It is convenient to refer here to the skulls of a Neanderthaloid appearance which have been found in Africa and Java. The former is represented by a skull from Northern Rhodesia, discovered in 1921, to which the name *Homo rhodesiensis* has been given, and the latter by a series of eleven crania which were found in 1931 and 1932 in Java, and which have been referred either to a new genus, *Javanthropus*, or, to a new species, *Homo soloensis*. It has been emphasized by several anatomists that in a number of features these skulls show differences from the classical Neanderthal type of Europe. Nevertheless, in their main characters they conform in a general way to this type, and indeed in certain respects, as in the supraorbital torus and the massive development of the ridges on the occipital bone for the attachment of the nuchal muscles, they have developed typical Neanderthal features to an exaggerated degree. It therefore seems reasonable to regard them simply as variants of the Neanderthal type.

The Rhodesian skull was found in unstratified detritus filling a cavern, and there is no adequate geological evidence by which its antiquity can be assessed. With it were found some limb bones of a modern human type,[1] but it remains uncertain whether these can be properly associated with the skull. The endocranial capacity is estimated at 1280 c.c.

The Javanese skulls all lack the facial skeleton. In their general contour and in the details of the frontal and occipital regions they show quite a close resemblance to the Rhodesian skull (Oppenoorth, 1932*a*, *b*, 1936, 1937). The temporomandibular joint is of modern human type. The capacities of three of the skulls are estimated at about 1160, 1300 and 1190 c.c. Only one limb bone has been found with the skulls, a tibia, and this is similar to that of modern man, showing none of the typical Neanderthal characters. The skulls were found at the village of Ngandong on the Solo river, about 10 km. north-east of Trinil. They were

[1] It has been stated, on the basis of a portion of the os innominatum, that the posture and gait of Rhodesian man were altogether peculiar. This conclusion, however, was later shown to be the result of an error in reconstruction (Le Gros Clark, 1928).

embedded in a mass of hard travertine composed of fluvial sand and grit impregnated with lime, and with them were found some problematical implements made of bone or stag-horn and some stone balls. The age of the Ngandong skulls remains uncertain. Oppenoorth believes the deposits to date from the middle Pleistocene. On the other hand, v. Koenigswald (1935), on the basis of the associated fauna, refers them to the late Pleistocene, while the late Prof. Callenfels (1936) believed they may be even as recent as the Mesolithic.

## V. PRE-MOUSTERIAN MAN

Reference has been made above to the Ehringsdorf skull, the antiquity of which is judged to be either early Mousterian or pre-Mousterian. Another skull of somewhat similar type was found at Steinheim in 1933. The faunal evidence

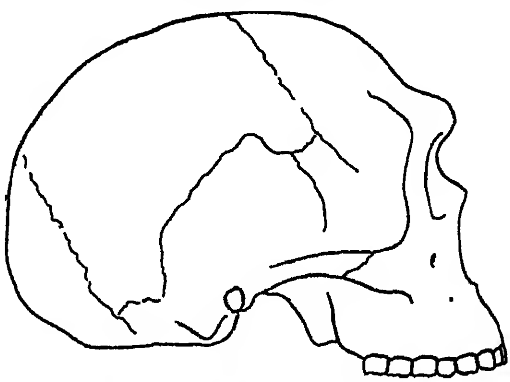

Fig. 11. Lateral view of the Steinheim skull. × 3/8. (As restored by Weinert, 1936.)

indicates that the Steinheim skull is of pre-Mousterian age and dates from the warm period of the Riss-Würm interglacial. The characters of the skull are instructive, for while it shows a fairly strong development of the supraorbital ridges approximating to the condition found in typical Neanderthal skulls, in other features, such as the rounded occipital region, the vertical sides of the skull, and the contour of the facial surface of the maxilla, it resembles more closely *Homo sapiens*. The cranial capacity, estimated at 1070 c.c., is also considerably below the average capacity of Neanderthal skulls (Weinert, 1936).

In 1935 and 1936 two portions of a human skull were discovered at Swanscombe in Kent. They were found *in situ* in well-stratified gravels of the 100 ft. terrace of the Thames. The importance of this fossil lies in the fact that its antiquity is extremely well attested by stratigraphical, faunal and archaeological evidence (Swanscombe Committee Report, 1938). It can be referred quite definitely to the middle Acheulian phase of Palaeolithic culture, and it probably dates from the Mindel-Riss interglacial period. The bones (occipital and left parietal) are well preserved, and apart from their thickness appear to be closely similar to those of modern man. They certainly show no distinctive Neanderthaloid features. Nothing is known of the face and jaws, and it remains possible that these may have been

strongly developed. Indeed, there are indications that this was probably the case, for an impression of the sphenoidal air-sinuses on the basi-occipital bone suggests that the accessory nasal air-sinus system was rather extensive. Nevertheless, the fact remains that, so far as can be judged from the dimensions and indices of the back part of the skull, the Swanscombe skull is astonishingly similar in all its main metrical characters to the average of available series of modern skulls. The height of the skull (basi-bregmatic) and the maximum bi-parietal width are indeed rather greater than the corresponding measurements of female British skulls. The cranial capacity is estimated at about 1325 c.c., which is well within the range of variation of modern man. The endocranial cast, also, provides evidence of quite a richly

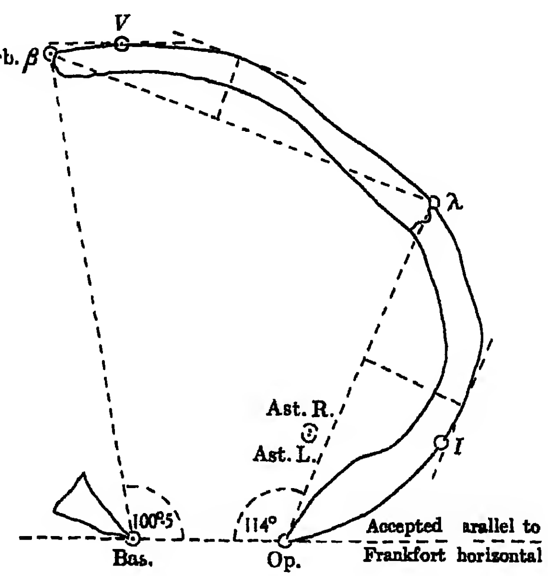


Fig. 12. Median sagittal contour of the Swanscombe parietal and occipital bones. × 1/2 *circa*. (From the Swanscombe Committee Report, 1938.)

convoluted brain which shows no appreciable difference from modern human brains.

The Swanscombe skull seems to establish without doubt that Acheulian man approximated to *H. sapiens* very much more closely than did the Neanderthal types of later Mousterian age. The specimen therefore confirms the evidence of the Steinheim and Ehringsdorf fossils, and also the evidence of the early Mousterian types. It must certainly be admitted that more palaeontological data are necessary before a final decision on this problem can be made, but at least it is reasonable on the evidence now available to assume that either *H. sapiens* or his immediate precursors were already in existence in pre-Mousterian times, and that the extreme Neanderthal types cannot therefore represent a transitional stage in the evolutionary origin of modern man.

## VI. THE ORIGIN OF *HOMO SAPIENS*

At the end of the Mousterian phase of Palaeolithic culture Neanderthal man disappeared—apparently with some abruptness. In deposits of later date the skeletal remains of man are all similar in their anatomical characters to the European populations of to-day. Whence did these modern types of man come? With the evidence at present available the answer to this question can only be conjectured. We have noted that in Acheulian times there were living in Europe men who were physically at least closely akin to *Homo sapiens*. There is little doubt, indeed, that they were the direct precursors of modern man.

Between Acheulian man and the early Pleistocene representatives of the *Pithecanthropus* group there is a considerable morphological hiatus. It has already been indicated, however, that the *Pithecanthropus* group showed a remarkable variability, the cranial capacity in some cases reaching well within the range of variation in modern man. It may be provisionally inferred that with a progressive growth of the brain the *Pithecanthropus* type would certainly have led to the appearance of a generalized Neanderthaloid type.[1] At this point in the evolutionary history of the Hominidae two separate lines of development evidently made their appearance. In one of these the expansion of the brain was associated with an exaggerated development of the supraorbital ridges, the jaws, palate and teeth, and certain retrogressive changes in the limb skeleton, leading eventually to the extreme Neanderthal type of later Mousterian date. In the other line a progressive enlargement of the brain was associated with a recession of the supraorbital ridges and the jaws, a diminution in the size of the teeth, the construction of a more evenly rounded cranium with a vertical forehead, and the retention of the limb characters already developed in the *Pithecanthropus* group. This second line evidently led through Acheulian man to *Homo sapiens*.

Undoubtedly the most serious gap in the palaeontological sequence of man still exists in the presumed transformation of the man-like apes of Miocene time into the ape-like men of the early Pleistocene. The former, in spite of the "progressive" tendencies which they showed in their teeth and jaws, were definitely apes, while the latter, in spite of the retention of a number of primitive features, were true men. What is most urgently needed now to fill this gap is information regarding the skull and limbs of those Miocene and Pliocene fossils which are at present referred to *Dryopithecus* and allied genera.

## VII. SUMMARY

Palaeontological evidence bearing on the evolutionary origin of the Hominidae is provided by dryopithecine fossils of Miocene and Pliocene date. These fossils consist almost entirely of jaws and teeth. They indicate that, while the dentition of *Dryopithecus* was essentially simian in its general characters, in certain features, notably

[1] Some authorities have argued with good reason that it is not practicable to insist on a taxonomic distinction between the *Pithecanthropus* group and Neanderthal man. See Zuckerman (1931).

the cusp-pattern and proportions of the molars, it showed some significant approach to a human type of dentition. In certain allied genera, this approach is still further emphasized by the conformation of the dental arcade. The fact that in some species of these fossil apes the characteristic specializations of modern anthropoid apes were already evident in incipient form suggests that the divergence of the evolutionary line leading to the Hominidae from that which culminated in the modern genera of anthropoid apes must probably be referred to the beginning of Miocene times.

More recent fossil apes from South Africa, even though they themselves may not bear any ancestral relation to man, emphasize the evolutionary potentialities of the Dryopithecinae for development in the direction of the Hominidae. However, a considerable gap still exists between the dryopithecine apes and the earliest known representatives of the Hominidae, a gap which can only be filled by further palaeontological discoveries, with particular reference to the skull and limb characters of the former.

The accession of new palaeontological material of the *Pithecanthropus* group (including those fossils which have been referred to the genus *Sinanthropus*) has served to emphasize its hominid status. It is important to note that, despite many primitive features of the skull, brain and dentition, the limb bones of the *Pithecanthropus* group are closely comparable with those of modern man. It becomes clear that if the modern characters of the human limbs had already been acquired so early as the beginning of Pleistocene times, the point of divergence of the Hominidae from the Simiidae must have been correspondingly more remote.

The *Pithecanthropus* group almost certainly provided the basis for the development of later types of man. Of these, one is represented by the rather specialized Neanderthal type of later Mousterian date. That this is to be regarded as an aberrant line is indicated by the fact that fossil human remains of early Mousterian and pre-Mousterian date were less distinctively "Neanderthaloid", and more akin in their anatomical features to *Homo sapiens*. There seems little doubt that these fossils represent, as a group, the direct ancestors of modern man.

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## ADDENDUM

In a recent communication (*Nature, Lond.*, **144**, 1939, p. 926) v. Koenigswald & Weidenreich have called attention to further discoveries of *Pithecanthropus* in Java during last year. These discoveries comprise an upper jaw with a completely preserved nasal floor and palate and most of the teeth, and the posterior part of a calvarium including the entire skull base. The authors reach the conclusion that "*Pithecanthropus* and *Sinanthropus*. . . are related to each other in the same way as two different races of present mankind".

# THE HISTOGENESIS OF TISSUES SENSITIVE TO OESTROGENS

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(*Received* 20 *September* 1939)

CONTENTS



## I. INTRODUCTION

Recent advances in knowledge of reproductive physiology have made it necessary to abandon, step by step, the view that oestrogenic hormones are specific in their nature and action. In the years immediately following Allen and Doisy's isolation, in 1923, of a definite oestrogenic extract, the substance was customarily referred to either as the hormone of the ovarian follicle or as the female sex hormone, while its action was believed to be restricted to the female reproductive system. It soon became clear, however, that the ovarian follicle is not the only source of the hormone, and ideas of source specificity had ultimately to be rejected. Such, too, was the fate of a frequently urged belief that oestrogenic hormone is the only kind of sex hormone elaborated by the female organism.

This was only the beginning of the breakdown of our views on the specificity of the female sex hormone. Soon after oestrone was obtained in pure crystalline state in 1929, a number of naturally occurring oestrogens with different chemical constitutions were recognized. At the same time research from another direction revealed the existence of numerous synthetic compounds, unrelated to the natural oestrogens, but possessing oestrogenic activity. The final blow to ideas of specificity

came with the discovery that some androgens have oestrogenic powers, and with the clear demonstration that oestrogens are not sex-specific, but that they can act on the male as well as the female, and in both sexes not only on the reproductive system, but on tissues wholly unrelated to the Müllerian and Wolffian ducts.

Not surprisingly, attempts to systematize knowledge about the action of oestrogens have become fewer in recent years, and at the present time there is no comprehensive hypothesis which aims at relating the many responses that are induced by oestrogens. The presentation of such a statement is the purpose of the present review. It is put forward with no illusions about either the permanency or completeness of most general interpretations in this field of experimental biology, but in the hopes that it may for a time serve a purpose as a working hypothesis.

The known effects of oestrogenic stimulation, other than such physiological changes as alterations in uterine contractility and in vaginal *p*H, may be broadly classified under three main types of response. The first appears to be a property of most, if not all, tissues, and shows itself mainly in a change in water content (Zuckerman *et al.* 1939). With little doubt it represents a generalized vascular and metabolic effect of the kind that takes place so intensively in the sexual skin of monkeys (Krohn & Zuckerman, 1937; Aykroyd & Zuckerman, 1938*b*; Zuckerman, 1939; Ogston *et al.* 1939).

The second type of response may be regarded as comprising those widespread changes which result from the direct and indirect, and usually histologically recognizable, effects of oestrogenic stimulation upon various endocrine organs (e.g. anterior lobe of pituitary, adrenals, parathyroid). Included under this class of response are, for example, those changes that result from the stimulation of corpora lutea (e.g. Robson, 1939), and also the skeletal changes that occur in mice which have been treated with oestrogenic hormone[1] (Gardner, 1936; Gardner & Pfeiffer, 1938).

The third type of response takes the form of cellular growth, and is limited to certain tissues, e.g. the reproductive organs and the gums. Sensitivity which shows itself in this way is presumably due to some common property of the cells that react, and unless it be assumed at the outset that this cellular characteristic always develops independently, it is necessary to seek some other explanation for the fact that so many tissues have this property in common. One possibility is that many of the tissues which react by proliferation are developmentally related. It is with this hypothesis, which has been sketched in broad outline in preliminary notes (Zuckerman, 1936*a*, *b*), that the present article is concerned. The first task is necessarily to survey the field of the proliferative response.

[1] The latter changes are classified as an indirect reaction because they are closely similar to those which occur in pigeons (Pfeiffer & Gardner, 1938), where they are undoubtedly associated with changes in calcium metabolism, apparently mediated through the parathyroids (e.g. Riddle & Reinhart, 1926; Macowan, 1932; Charles & Hogben, 1933; Riddle & Dotti, 1936; Altmann & Hutt, 1938). In the lack of a contrary hypothesis, and in spite of the negative observation of Levin & Smith (1938) that oestrogenic stimulation has little effect upon serum calcium in rats, rabbits and monkeys (a finding at variance with that of Riddle & Dotti (1936, 1938) and that of Cheymol & Quinquaud (1938)), it may therefore be presumed, at present, that the skeletal changes which are effected in mammals by oestrogens are mediated as in birds.

## II. REACTING TISSUES

### (1) *Female*

#### (*a*) *Fallopian tubes.*

The only changes which oestrogenic stimulation normally produces in the tubes are epithelial proliferation, with multiplication of the mucosal folds, and secretion. Massive and prolonged oestrogenic treatment may lead to considerable growth of the tube, with hyperplasia[1] of its epithelial lining, from which dilated glandular offshoots penetrate the muscular coat (Lacassagne, 1935*a*).

#### (*b*) *Uterus.*

The gross effects of oestrogenic stimulation on the uterus vary in different species, but the essential change is the same in each, namely, fibromuscular and epithelial proliferation. The epithelial change is in almost all species associated with the proliferation of tubular endometrial glands, which in the primate uterus provide the characteristic "interval" picture.

Excessive oestrogenic stimulation may lead either to cystic glandular hyperplasia of the endometrium (with irregular penetration of the hypertrophying myometrium by the hyperplastic glands), or to a process of "metaplasia" in which the normal secretory cylindrical epithelium of the cavum uteri and glands becomes transformed into stratified squamous epithelium.[2] The first of these changes has been observed, under experimental conditions, in a mangabey monkey (*Cercocebus torquatus atys*) and chimpanzee (*Pan satyrus*) (Zuckerman & Morse, 1935); a marmoset (*Hapale jacchus*) (Russell & Zuckerman, 1939); rhesus monkeys (Zuckerman, 1937*c*; Engle & Smith, 1938); and in mice, guinea-pigs, rabbits and rats (e.g. Burch *et al.* 1931; Parkes, 1935; Lacassagne, 1935*a*; McEuen *et al.* 1936; Nelson, 1937; Lipschütz, 1937). The second change occurs throughout the uterus in rats (e.g. Selye *et al.* 1935), mice (Gardner *et al.* 1936) and rabbits[3] (Pierson, 1938), but only in the cervix of guinea-pigs[4] and rhesus monkeys, close to the line of junction of vaginal and uterine epithelium (Lipschütz, 1937; Overholser & Allen, 1933; Engle & Smith, 1935; Zuckerman, 1937*a*). This region in the rhesus monkey is normally an unstable zone of transition between cylindrical and stratified squamous epithelium, for it contains foci of "metaplasia" even in untreated animals (Sandys & Zuckerman, 1938).

[1] By "hyperplasia" is meant the overgrowth of tissues due to the excessive proliferation of their cellular components.

[2] "Metaplasia" is the term loosely used to describe any apparent transformation of one type of well-characterized tissue into another. It has been widely applied to the particular change under consideration here. Whether or not this change actually comes within the scope of a strict definition of the term is discussed below (p. 249).

[3] Neither Lacassagne (1935*a*) nor Zondek (1936), who have also investigated the effects of prolonged and intensive stimulation in rabbits, has observed these changes.

[4] According to Nelson (1939), while the changes in the guinea-pig are mostly confined to the cervix, they may extend to the fundus uteri and even into the proximal parts of the cornua.

(*c*) *Vagina.*

Oestrogenic stimulation leads to proliferation of the stratified vaginal epithelium, resulting in cornification and desquamation of the more superficial layers of cells. Very small doses of oestrogenic hormone lead, on the other hand, to partial mucification of the vaginal epithelium, full mucification being an effect of the action of balanced amounts of oestrogenic and progestational hormone (Klein, 1937).

(*d*) *Genital and circumgenital skin.*

Like that of the vaginal wall, the epithelium of the vulva of monkeys and, in many species, of the surrounding genital or sexual skin, undergoes proliferation under the influence of oestrogenic stimulation. The limits of the reactive zone vary in different species of monkey (Zuckerman, 1930, 1937*b*), and at different times (Zuckerman *et al.* 1938), but under normal physiological conditions the most responsive region is the vulva itself (p. 255).

Glycogen can be demonstrated in all the epithelium of the female and male urogenital tract that undergoes proliferation in response to oestrogenic stimulation, including that of the genital and circumgenital skin (Carleton & Zuckerman, 1939).

(*e*) *Muscular and connective tissue of reactive zones.*

The muscular tissue of the female reproductive organs, derived presumably from the mesoderm of the intermediate cell mass and genital cord, hypertrophies, partly, although slightly, by mitotic division, under the influence of oestrogenic stimulation (see Reynolds, 1939; Crandall, 1938). So, too, do the connective tissue cells of the endometrium (Crandall, 1938), of the submucosa of the vagina, and of the sexual skin (Aykroyd & Zuckerman, 1938*b*). The increase in size of the connective tissue cells during oestrogenic stimulation appears to be associated with oedema of the reacting tissues (Aykroyd & Zuckerman, 1938*b*).

Excessive stimulation may lead in rabbits and guinea-pigs to the development of fibromyomatous growths in the uterus (Lacassagne, 1935*a*; Nelson, 1937; Moricard & Cauchoix, 1938). The occurrence of tumours in non-gential regions is noted below.

(*f*) *Mammary glands.*

Oestrogenic stimulation causes growth of the nipples and duct system of the mammary glands of both female and male mammals. In some species, e.g. the guinea-pig (Turner & Gomez, 1934), it may induce true alveolar formation. This has also been claimed in the monkey (Gardner & van Wagenen, 1938), but the finding was not confirmed in a study of male monkeys in which stimulation was continued for over a year (Folley *et al.* 1939). On the other hand, secretory alveoli did form as a result of oestrogenic stimulation in two of four spayed female rhesus monkeys whose ovaries were removed before luteinization had occurred.

In cancer-strain mice the proliferative effects of oestrogenic stimulation may proceed so far that mammary adenocarcinomata develop (see Lacassagne, 1937; Gardner, 1939). Metaplasia leading to the differentiation of sebaceous glands has also been reported in such animals (Florentin & Binder, 1939).

(*g*) *Nasal mucosa and gums.*

The nasal mucosa of rhesus monkeys, particularly that of the middle and inferior turbinates, responds to oestrogenic stimulation in the manner of the sexual skin (Mortimer *et al.* 1936; 1936*a*, *b*). Castration causes regressive changes in the mucosa (Champy, 1930). Histological observations of the former changes are not recorded, but similar stimulation leads to considerable epithelial growth, with superficial cornification, of the gums (Ziskin *et al.* 1936).

(*h*) *Feathers.*

Oestrogenic hormone has a pronounced effect upon the growth of feathers, in many species being responsible for the plumage that is characteristic of the female. According to Parkes & Emmens (1939), in every case in which ovarian hormones determine the feathering of a hen, growing feathers in the cock or capon bird can be changed to the female type by the administration of an adequate dose of oestrogen.

It may be noted that there are some indications that hair cycles in female mammals (rats, ferrets) may be connected with the ovarian cycle (Butcher, 1934; Bissonnette, 1935).

(2) *Male*

(*a*) *Mice and rats.*

The epithelium of the coagulating glands of the mouse prostate undergoes hyperplasia and stratified squamous metaplasia under the influence of oestrogenic stimulation (Lacassagne, 1933; de Jongh, 1933; Burrows & Kennaway, 1934; de Jongh, 1935; Burrows, 1935*a*, *b*). The changes which occur in the male mouse are not, however, confined to just this region of the prostate. According to Burrows (1935*b*), the first organ that responds in this way is a cyst which is occasionally found in the situation where the urethral end of Müllers duct would be expected to occur. Then in order of response come the coagulating glands, the seminal vesicles, the periurethral glands (including Cowper's glands) and the entire urethra, the other lobes of the prostate, the vas deferens and, finally, the epididymis. In all the organs which open into the urethra, the change begins in the urethral parts of the collecting ducts and spreads peripherally into the organ. When oestrogenic treatment is stopped, "recovery takes place in the reverse order to that in which the pathological changes occur".

These changes, which also occur in the absence of the pituitary (de Jongh, 1934), are associated with fibro-muscular growth in the reacting organs (Freud, 1933; Korenchevsky & Dennison, 1934–1935; Overholser & Nelson, 1935; de Jongh, 1935; Korenchevsky *et al.* 1936).

While fibro-muscular growth is almost as marked in the rat as in the mouse, the metaplastic changes which result from oestrogenic stimulation are more limited (de Jongh, 1935; Weller *et al.* 1936).

As noted above, oestrogenic stimulation leads to considerable proliferation of the duct system of the mammary glands in male mice and rats, as well as in other male mammals.

(*b*) *Monkeys and man.*

(i) *Prostate.* The most striking change which oestrogenic stimulation causes in the prostate of monkeys is growth and stratified squamous metaplasia of the "uterus masculinus" (Parkes & Zuckerman (1935) and van Wagenen (1935), working on the rhesus monkey, *Macaca mulatta*; Parkes & Zuckerman (1935), on the Guinea baboon, *Papio papio*; and Courrier & Gros (1935*b*), on the Barbary ape, *Macaca inuus*). An increase in the size of the whole organ is usually not apparent during short periods of oestrogenic stimulation, but is very evident when treatment is continued for a year or more (Zuckerman, 1938*a*; Zuckerman & Sandys, 1939). The change appears to be mainly due to growth of the fibro-muscular elements of the "middle lobe" (prespermatic lobe or "utricular bed") of the prostate.

Observations on other species of monkey—the common marmoset (*Hapale jacchus*), the brown capuchin (*Cebus fatuellus*), the common macaque (*Macaca irus*), the green monkey (*Cercopithecus aethiops sabaeus*), the drill (*Mandrillus leucophoeus*), the mona monkey (*Cercopithecus mona*), and the hanuman langur (*Presbytis entellus*)—produced further interesting results (Zuckerman & Parkes, 1936). Injections of oestrone were continued for from 14 to 39 days. The first two species showed no changes except in the urethral epithelium (see pp. 237, 256); the changes in the third, fourth, fifth and sixth were the same as in the rhesus monkey; while in the Entellus langur the "uterus masculinus" had responded by glandular hyperplasia, a change which was confirmed in a later study in which injections were continued for 483 days (Zuckerman & Sandys, 1939). This glandular hyperplasia is completely different from the squamous metaplasia that occurs in other species of monkey, and in general takes the form of a proliferation of secretory acini.

It should be noted that the true prostatic glandular acini of monkeys, including those of the cranial lobules which van Wagenen (1936) has suggested are homologous with the coagulating glands of mice, do not undergo hyperplasia or metaplasia in response to oestrogenic stimulation (Zuckerman, 1938*a*; Zuckerman & Sandys, 1939). Other changes do, however, take place in the prostate when oestrogenic stimulation is prolonged. Stratified squamous metaplasia occurs in the terminal parts of both the main collecting ducts of the prostate and the common ejaculatory ducts, and also in the lower part of the prostatic urethra. The part cranial to the opening of the uterus masculinus and ejaculatory ducts remains practically unchanged. The extent of the urethral change varies in different species.

There are few observations on the effect of oestrogenic stimulation on the human prostate. Hamilton *et al.* (1937) report no changes in mature men, but Moore & McLellan (1938) found that stimulation results in increased stratification of the urethral transitional epithelium, and foci of stratified squamous metaplasia appear both in the epithelium of the urethra and that of the prostatic collecting ducts. These changes are similar to those which are normally observed in the new-born male infant (Aschoff, 1894; Schlachta, 1904; Sharpey-Schafer & Zuckerman, 1939). The changes in the infant prostate, which are presumably due to leakage of oestrogenic hormone from the maternal organism, begin during the

seventh or eighth month of gestation, and show themselves by stratified squamous metaplasia of the collecting ducts (of mainly the upper part of the prostate), of the epithelium of the uterus masculinus, and of the epithelium over the summit of the crista urethralis. They may persist until two months after birth (Schlachta; Sharpey-Schafer & Zuckerman). In some cases the change in the uterus masculinus appears to be intermediate between the complete squamous metaplasia which is induced by oestrogenic stimulation in the uterus masculinus of the rhesus monkey, and the glandular hyperplasia which was observed in the langur.

(ii) *Seminal vesicles.* Oestrogenic stimulation in immature monkeys leads to a considerable increase in the size of the seminal vesicles. The change is due to fibro-muscular growth, the epithelial elements of the vesicles becoming reduced. If stimulation is prolonged, the vesicular lobules become converted into thick-walled muscular tubes lined by a simple non-folded epithelium. Epithelial hyperplasia and metaplasia have not been observed in the seminal vesicles of monkeys. They occur, however, in the terminal parts of the ejaculatory ducts.

(iii) *Vasa deferentia.* Epithelial hyperplasia and metaplasia were not observed in the vasa deferentia of rhesus macaques and Entellus langurs, which were injected with oestrogenic hormone for a year and longer.

(iv) *Hydatids of Morgagni.* Oestrogenic stimulation leads to considerable growth of the hydatids of Morgagni, the change affecting both the stromal and epithelial elements. Stratified squamous metaplasia has not been observed, the hyperplasia being glandular in nature (Zuckerman & Krohn, 1937).

(v) *Urethra.* Hyperplasia (in many cases leading to squamous metaplasia with superficial cornification and desquamation) of the epithelium of the summit of the crista urethralis has been observed in all the male primates whose prostatic reactions to oestrogenic stimulation have been tested. In some species (e.g. the langur, *Presbytis entellus*; the marmoset, *Hapale jacchus*; the brown capuchin, *Cebus fatuellus*; and man, *Homo sapiens*) no further changes appear to occur. In others (e.g. the rhesus monkey, *Macaca mulatta*; the drill, *Mandrillus leucophoeus*; the Guinea baboon, *Papio papio*; the Barbary ape, *Macaca inuus*; the green monkey, *Cercopithecus aethiops sabaeus*; and the mona monkey, *C. mona*) the changes extend from the region of the urethral opening of the uterus masculinus and ejaculatory ducts to the external opening of the urethra. With the exception of the lining of the upper prostatic urethra, which remains practically unchanged, the whole of the urethral mucosa may be transformed into a stratified, cornified and desquamating epithelium. In the prostate the metaplastic process is best marked on the dorsal wall of the urethra, the ventral wall in some cases being unchanged.

(vi) *Cowper's glands.* No hyperplastic or metaplastic changes were observed either in the acini, the smaller collecting ducts, or the main collecting ducts of Cowper's glands in any of seventeen rhesus monkeys which were injected with oestrone (Aykroyd & Zuckerman, 1938*a*). Two of these animals had been under treatment for a year. No changes are to be seen in Cowper's glands of newborn human infants (Sharpey-Schafer & Zuckerman, 1939).

(vii) *Sexual skin.* The genital and circumgenital skin of male monkeys, in species in which the female undergoes sexual skin changes, usually responds to oestrogenic stimulation in the way the corresponding skin of the female does (Dohrn *et al.* 1933; Bachman *et al.* 1935; Zuckerman & van Wagenen, 1935; Courrier & Gros, 1935*a*; Zuckerman *et al.* 1938).

(*c*) *Other mammals.*

(i) *Dog.* The urogenital tract of the dog is very sensitive to oestrogenic stimulation (de Jongh & Kok, 1935; Zuckerman & Groome, 1937; de Jongh *et al.* 1938; Petit *et al.* 1939). Not only the uterus masculinus and collecting ducts, but also the secreting acini of the prostate undergo stratified squamous metaplasia, until the glandular system is converted into a series of cysts, each filled with a core of desquamated epithelial cells. The entire urethra also undergoes squamous metaplasia, the upper prostatic urethra apparently being as sensitive as the rest of the tract.

The bladder, vasa deferentia and epididymis also increase in size as a result of oestrogenic stimulation (de Jongh *et al.* 1938). No epithelial changes have been recorded in these organs, and their growth is presumably due to proliferation of fibro-muscular elements, as de Jongh and his co-workers also suggest it is in the case of the rat epididymis (Sindram *et al.* 1939).

(ii) *Ground squirrel,* Citellus tridecemlineatus. Oestrogenic stimulation causes stratified squamous metaplasia, with superficial cornification, in the epithelium of the following parts of the urogenital tract of the male ground squirrel (Wells, 1936): bulbar gland, Cowper's glands (collecting tubules and main ducts only), main prostatic ducts. Squamous metaplasia, without cornification, was observed in the collecting ducts of the prostate. Fibro-muscular growth occurred in the seminal vesicles, epididymis, and ductus deferens, but no epithelial changes were observed in these organs in experiments in which stimulation was continued for 34 days.

(iii) *Guinea-pig.* The uterus masculinus of castrated guinea-pigs undergoes stratified squamous metaplasia as a result of oestrogenic stimulation (van der Woerd, 1936; Courrier & Cohen-Solal, 1936; Laqueur, 1936). The reaction is somewhat modified in the presence of the testes.

(iv) *Cat.* Oestrogenic stimulation causes a great increase in the size of the prostate and bulbo-urethral glands (Courrier & Gros, 1938; Starkey & Leathem, 1939; Gros, 1939). This is due mainly to stimulation of normal secretory activity in the epithelial elements of these organs, but stratified squamous metaplasia occurs in the urethra, the uterus masculinus, and some secretory glandular acini. Raynaud (1937*a*) has shown that very profound changes also take place in the bladder and urethra of the oestrogen-treated cat, the muscular coat of the former hypertrophying, and the epithelium in the region of its neck and that of the entire urethra and peri-urethral glands undergoing stratified squamous metaplasia. Even more profound changes in the vesical epithelium have been reported by Lacassagne (1935*b*) in mice which had received large doses of oestrogen, squamous metaplasia of the

mucosa being associated with papillomatous outgrowths and epithelial ingrowths that reached as far as the muscular coat. Lacassagne has observed similar metaplastic changes in the urethra of female mice.

### (3) *Other responses*

Pierson (1938) has reported the occurrence of considerable changes in the stomach of a single rabbit that was treated with oestrogenic hormone for 3 years. A layer of striated muscle had developed immediately under cover of the serosa, and between this layer and the smooth muscle was a layer of tissue resembling mammary gland. No such changes have been reported by any other worker.

It has been noted above that oestrogenic hormone stimulates growth of muscle, and occasionally the development of tumours, within the reproductive tract of male and female animals. More rarely it provokes abnormal fibro-muscular growth and tumours elsewhere in the body. Thus Koref *et al.* (1939) report the occurrence of fibrous tumours in the abdominal cavity of both male and female guinea-pigs, and Vargas & Lipschütz (1938) and Lipschütz *et al.* (1938) state that extragenital tumours may appear even before uterine growths in the castrated female. These extragenital tumours are fibromas or fibromyomas, and they may invade smooth and striated muscle, pancreas and liver.

Non-genital sarcomata, usually at the site of injection, have occasionally been reported in cancer-strain mice receiving oestrogens in oil, but similar tumours have also been reported in control mice receiving only the oil solvent (see Allen *et al.* 1939).

## III. THE CHARACTER OF THE PROLIFERATIVE RESPONSE

Consideration of all these changes makes it clear that, under normal physiological conditions, oestrogenic sensitivity manifests itself histologically both in the orderly proliferation of secretory cells (for convenience this response is referred to below as "glandular"), and in the proliferation, cornification and desquamation of stratified squamous epithelium (for convenience called "squamous response"). These two types of response also appear under abnormal conditions of stimulation—for example, during prolonged and intense treatment with oestrogenic hormone—but whereas each is characteristic of definite tissues under normal conditions, tissues which usually undergo orderly glandular proliferation (e.g. the endometrium of the rat) may become metaplastic under non-physiological conditions, and transformed into proliferating stratified squamous epithelium. The occurrence of metaplastic changes is not only dependent on the duration and intensity of stimulation, but also, to some extent, on the age of the animals subjected to treatment (de Jongh & van der Woerd, 1939).

Whether or not there is a histochemical relation between the two types of response,[1] the glandular is, in the main, confined to tissues (e.g. uterus, hydatid of Morgagni) that are undoubtedly derived from the Müllerian duct. Exceptions are the mammary glands, which originate in the mammary ridges (themselves

[1] See quotation from Suntzeff *et al.* (1938), p. 251.

derived from ectoderm) and the prostate of the cat. The squamous response, on the other hand, occurs in tissues that are believed to have widely different origins, e.g. the vagina and the uterus masculinus, which are presumed Müllerian duct derivatives; the prostate, Cowper's glands, and peri-urethral glands, which are derived from the urogenital sinus, and are generally presumed to be entodermal; the bladder of the mouse, and the base of the bladder in the cat, which are entodermal, and in the case of the latter probably Wolffian as well; the circumgenital skin and feathers, which are ectodermal derivatives, as are also the nasal mucosa and gums, derived from the stomadoeum; and finally the seminal vesicles, the vas deferens, and the epididymis in the mouse, which are Wolffian duct derivatives.

If these commonly believed embryological derivations are correct, it follows on the one hand that Müllerian derivatives are capable of both the glandular and squamous types of response, and on the other, that the squamous response occurs in tissues which have almost every kind of embryological origin. If such is indeed the case, no useful anatomical explanation can be offered for the responses of tissues to oestrogenic stimulation. This, however, is not the usual view, and several suggestions have been put forward to explain the morphological and histogenetic basis of the responses. Lacassagne (1933), who was concerned only with the dorsal lobes of the mouse prostate, suggested, for example, that these structures may be derivatives of the Müllerian duct—the basis for this suggestion undoubtedly being the view, widely held until recently, that the action of oestrogenic hormone is mainly limited to Müllerian structures. Burrows, again (1935*a*), suggested the possibility that the Müllerian ducts may contribute to the formation of the part of the urogenital sinus into which they open, for from this part of the sinus arise many of the prostatic ducts which respond to oestrogenic stimulation. But because other tissues that are clearly not Müllerian in origin also react, he later (1935*b*) qualified this interpretation and stated his views as follows: "the effects of an oestrogen on mice of either sex are almost or entirely confined to those organs, including the mamma and preputial glands, which are concerned directly or indirectly with reproduction. Structures which have no active part in reproduction may yet respond to an oestrogen if morphologically they are components of the reproductive system in either sex, or have their embryological origin in that system." A similar view was advanced by de Jongh *et al.* (1938).

This interpretation, while it clearly defines the field of the oestrogenic response so far as the reproductive system is concerned, does not, however, either indicate the different developmental and anatomical complexities of the male and female reproductive organs, or provide any explanation for the different types of response which occur in different regions of the urogenital tract. Detailed analysis of the changes which oestrogens cause shows that it is possible to throw light on these two problems.

### (1) *The responses of true Müllerian tissue*

The first question that presents itself is whether or not both the glandular and squamous types of response occur in true Müllerian tissue, and the problem immediately resolves itself into a consideration of the origin of the vagina, for under

normal conditions the vagina is the only presumed derivative of the Müllerian ducts which exhibits the squamous response. Undoubted Müllerian derivatives, like the uterine tubes and the uterus, undergo only glandular proliferation under normal conditions. Evidence bearing on this problem may be adduced from several sources.

(*a*) *The origin of the vagina and uterus masculinus in man.*

It is generally agreed that early in development (at about the 30 mm. stage), the Müllerian ducts come into contact, at Müller's tubercle, with the epithelium of the urogenital sinus, into which, according to some authors (e.g. Wood-Jones, 1904), the conjoined ducts at this time actually open. All investigators describe the subsequent occurrence of a process of obliteration of the lumen of the fused ducts, beginning distally and proceeding cranially, with the consequent appearance of a utero-vaginal cord of cells canalized only in its upper part. Such a process of obliteration occurs in both sexes, in both of which the same uncertainties in our knowledge of the later developmental processes apply. These uncertainties mainly concern the derivation of the solid cord of cells that makes up the lower part of the utero-vaginal canal, and which is the rudiment of the vagina. Bloomfield & Frazer (1927) and Hunter (1930), agreeing with the older opinion, hold that all these cells in the female are definitely Müllerian in origin. Evatt (1909), Meyer (1909), Alesio (1929) and Vintemberger (1926), on the basis of their findings, state a corresponding view for the male. On the other hand, Spuler (1930) and Vilas (1932) present evidence that the cells are derived from the sinus epithelium over Müller's tubercle. Their view is that the caudal part of the utero-vaginal canal retrogresses, and that the canal retains connexion with the tubercle only through the medium of a cord of cells proliferated from the sinus. Vilas (1933) has also described an identical process in the male, whose Müllerian ducts, he believes, as a rule atrophy completely. In this his observations appear to tally with those of Perna (1924). In support of this view, it may also be noted that Meyer (1909), whose studies led him to the general conclusion that the uterus masculinus is derived from the caudal part of the Müllerian ducts, also described certain cases in which sinus epithelium invaded the foetal utriculus masculinus. The opinion stated by Koff (1933), on the basis of a careful investigation of the derivation of the solid vaginal plate, is an intermediate one. According to him, about the lower fifth of the vagina is derived from "sino-vaginal" bulbs proliferated from the squamous epithelium over the site of Müller's tubercle, while the upper four-fifths develop from the fused and solid caudal segment of the Müllerian ducts.[1]

Certain developmental abnormalities which crop up in clinical practice undoubtedly support the view that the vagina is either wholly or in part derived, not from the Müllerian ducts, but from the urogenital sinus. Thus Young (1937) reports several cases of male pseudo-hermaphrodites who had testes and male

[1] It may be noted that the belief that the Wolffian ducts may be concerned in the development of the lower end of the human vagina is not held by most recent students of the problem (e.g. Bloomfield & Frazer, 1927; Hunter, 1930; Vilas, 1932; Koff, 1933).

accessory organs, and who also possessed vaginae, up to 3 in. deep, opening at the surface of the body (but no other female internal reproductive organs). This condition is usually associated with severe degrees of hypospadias. Occasionally such male pseudo-hermaphrodites also possess a vestigial uterus and Fallopian tubes, and sometimes the vagina (which may be 3 in. long) opens into the posterior urethra. In the latter class of cases the vagina may justifiably be regarded as a very large "uterus masculinus".

One of these abnormalities has recently been found in the dissecting room of this department (Boldero & Zuckerman, 1939). The individual concerned was a presumed female aged 77 years. Her external form and genitalia were typically female, and she possessed a vagina, 6 cm. long, which ended blindly. No trace could be found of a uterus either by macroscopic or microscopic examination, nor were any definite male accessory organs discovered. Her gonads were inguinal in position, but so involuted and in such bad condition for histological study that it was impossible to tell microscopically what their previous character had been. Attached to them, on either side, were the fimbriae of the ostium abdominale of the Müllerian duct.

There can be no doubt that the most reasonable interpretation of such abnormalities is that the blind vagina is derived from the urogenital sinus. It is of course possible that the vagina develops from the caudal and separated end of fused Müllerian ducts, but in view of the embryological evidence cited above, and the further evidence noted below, it seems the less likely explanation.

In the reverse condition of female pseudo-hermaphroditism, associated with adrenal disorder, masculinization of the external genitalia may carry the urethra forwards so that the vagina opens into the urethra (Young, 1937). These observations are closely paralleled by those derived from experimental studies of intersexuality (see below). The condition in which the vagina opens into the upper part of a long closed urogenital canal is also reminiscent of the normal anatomy of the female spotted hyaena, *Crocuta crocuta* (Matthews, 1939).

(*b*) *The vagina of the mole*, Talpa europaea.

It is convenient to consider at this point the vagina of the mole, as its development and the changes which take place in it throw considerable light on the question of the participation of non-Müllerian elements in the formation of the organ.

The vagina of the mole is not patent in the non-breeding season, and during this period of the year the sexes can be distinguished only by differences in perineal dimensions (Matthews, 1935). The similarity of the external genitalia of the male and female mole is reflected in their apparently more or less identical embryonic development. Thus Wood-Jones (1914) found that in both sexes the opening of the urogenital sinus is carried forwards "by closure of the inner genital folds, to the tip of the genital tubercle, which then becomes secondarily ensheathed within the overgrowing outer genital folds".

Wood-Jones has shown that up to about the 30 mm. stage of embryonic life, the Müllerian ducts open into the urogenital sinus close to the Wolffian ducts.

When the urogenital sinus is carried forwards to the tip of the clitoris by the fusion of the inner genital folds, the connexion of the Mullerian ducts with the sinus becomes lost, and the ducts, though fused above to form a common chamber with a wide lumen, pass downwards to the central region of the perineum in the form of attenuated strands of cells. At the same time cords of ectodermal tissue grow in from the surface to meet the apparently degenerating Müllerian cells. This ingrowth is occasionally unilateral, and the ingrowing cord (or cords) is always crescentic in outline. During the breeding season the vaginal orifice is formed by canalization of the ingrowing epithelial cord (or fused cords), and at the end of the season the canalized cord once again becomes solid.

According to Matthews, the length of the solid distal epithelial strand varies from 2 to 5 mm. The length of the whole utero-vaginal canal, which is taken as the distance between the perineal site of the vaginal orifice and the junction of the utero-vaginal canal with the uterine cornua, varies from 13–25 to 36·50 mm.

It is noteworthy that the Müllerian component of the female reproductive tract, from which the uterus and tubes develop, is fully patent until some distance from the perineal surface, and that it is impossible to tell whether or not the whole epithelial cord of cells which connects the canalized Müllerian part of the tract with the surface is purely ectodermal in origin or both ectodermal and Müllerian. In either case it is perfectly clear that ectodermal (i.e. non-Müllerian) tissue makes a considerable contribution to the formation of the utero-vaginal canal.

(*c*) *Experiments on intersexuality in rats, mice and guinea-pigs.*

It was first observed by Dantchakoff (1937*a*, *b*, 1938*a*) that female guinea-pig foetuses can be altered in a male direction by injecting them *in utero* with testosterone propionate. This experimental procedure leads to the development of male accessory reproductive organs, and to the regression of the female organs. The external genitalia become indistinguishable from those of the normal male, and a vagina does not develop, although the body of the uterus may be of normal size. Similar changes have been observed in the female young of rats and mice injected during pregnancy with androgenic hormone (Green & Ivy, 1937; Green *et al.* 1938*b*; Raynaud, 1937*b*, *c*; 1938*a*, *b*, *c*; Hamilton & Gardner, 1937; Hamilton & Wolfe, 1938). In such experiments small doses of androgen inhibit the development of all but the proximal part of the vagina, while adequate dosage results not only in complete suppression of the lower part of the vagina, but also in the appearance of fully masculinized external genitalia. The uterus is never suppressed.

A very clear picture of the change is given by Raynaud. In the newborn normal female mouse the lower end of the uterus forms a single solid cord, which comes into contact with the urethra, and then continues distally, enlarging and becoming triangular in cross-section as it proceeds to the dorsal and separated part of the urogenital sinus, with which it has already made contact at a higher level. The solid vaginal cord is regarded by Raynaud as originating from the urogenital sinus. In the normal newborn male, on the other hand, the only part of the utero-vaginal canal which persists is a small utricle whose junction with the dorsal wall of the

upper part of the urogenital sinus, from which it is wholly derived, is in the form of two solid cords. The dorsal part of the urogenital sinus does not differentiate to form the distal part of the vagina.

In the masculinized newborn female the mucosa of the distal part of the uterus is pseudo-stratified and unlike Müllerian epithelium. The uterine lumen divides into two, each lumen representing a Müllerian duct. These in turn become solid and join the urethra as in the male.

A similar absence of the distal part of the vagina has been reported in two intersexual ground squirrels, *Citellus tridecemlineatus* (Wells, 1937). In one the proximal part of the vagina opened into the membranous urethra, and in the other it ended blindly. In the intersexual shrew (*Sorex minutus*) described by Brambell & Hall (1936), it ended in the left vas deferens, which in turn joined the right, to form a common efferent duct.

Experiments in which the male foetuses of rats and mice are feminized by the injection of oestrogenic hormone into the pregnant mother have yielded equally important data. Thus Greene *et al.* (1938*a*) found that the development of the male accessory organs is partially suppressed, and that the newborn male possesses a vagina "comparable in development to that found in the normal newborn female". In similar experiments Raynaud (1939*a–f*) found that not only the vagina, but also the uterus may in some cases develop.

In so far as the same experimental procedure may lead to the presence of the one and the absence of the other, it is perfectly plain from these observations that the upper and lower parts of the utero-vaginal canal represent different developmental entities, only the upper, presumably, being Müllerian in origin.

### (*d*) *The histogenesis of the vagina in the rat, mouse and guinea-pig.*

Raynaud's very detailed experimental and histological observations make it clear that the vaginal mucosa of the mouse, is in greater part, if not entirely, derived from the epithelium of the urogenital sinus. According to this observer, the cytological characters of the cells in the solid cord which forms the distal part of the utero-vaginal canal, and which becomes the definitive vagina, are those of urogenital sinus epithelium, and not of epithelium derived from the Müllerian ducts. Greene *et al.* (1938*a*) take the same view about the lower part of the vagina of the rat, while according to Dantchakoff (1937*b*) the typical basophilic Müllerian epithelium of even the lower part of the uterus of the foetal guinea-pig becomes transformed to a clear epithelium similar to that of the urogenital sinus. It is interesting, too, that Meyer (1910), who studied human material, states that the boundary between uterine and vaginal epithelium always lies at first in the cervical canal, and that as foetal life progresses, the epithelium that differentiates into cylindrical cells "destroys the pavement epithelium as far as the external os uteri, and in about one-third of cases even further".

It would seem safe to conclude from these observations that the epithelium of the vagina in rats, mice and guinea-pigs is derived in greater part, if not wholly, from the urogenital sinus. It is obvious that the fact that the vagina in the pre-

pubertal rat and mouse is a solid cord of cells, entirely different histologically from the uterus, accords better with this interpretation than with the alternative hypothesis that both are derived from the Müllerian ducts.

It is convenient to refer in this section to an interesting observation which suggests that the "uterus masculinus" in Cetacea does not arise from the Müllerian ducts. This observation was made by Meek (1918) on a specimen of the white-beaked dolphin (*Lagenorhynchus albirostris*). In this animal the Müllerian ducts had not completely disappeared, but they were altogether unrelated to the "uterus masculinus", which was normal in disposition.

### (*e*) *The vagina in the monkey.*

Reliable information about the development of the vagina in monkeys is not available. Observations on the effect of oestrogenic stimulation on several species of monkey suggested, however, that it and the "uterus masculinus" are derivatives of the urogenital sinus and not of the Müllerian ducts (Zuckerman, 1936*a*, *b*). It was found later that the vagina in one species of monkey, *Cercopithecus aethiops sabaeus*, the green monkey, is a solid cord until puberty (Zuckerman, 1938*b*). In the youngest specimens examined the cord was patent only immediately above the opening of the urethra, and in the region of the fornices. Experiment showed that it could be completely canalized by means of oestrogenic stimulation. A similar instance of non-canalization of the vagina has also been encountered in a rhesus monkey (*Macaca mulatta*).

The resemblance of this condition to that in the prepubertal mouse is complete, and *a priori* it would be expected that the embryological origin of the solid cord would be the same (i.e. from the urogenital sinus). It is therefore of interest that the "uterus masculinus" in monkeys may sometimes show a very close resemblance to the structure which bears the same name in the mouse.

It was noted above that the "uterus masculinus" of the infantile mouse bifurcates at its point of contact with the urogenital sinus, from which it is derived. The "uterus masculinus" of the rhesus monkey is usually unilocular, but in four of a series of fifty-eight specimens it proved to be double, as in the mouse, consisting of separate ducts which opened independently, on either side of each other, on the summit of the crista urethralis. The separated "halves" of the "uterus masculinus" were crescentic in cross-section, and this finding is of particular interest in so far as it is reminiscent of the ectodermal cord of cells which forms the distal part of the utero-vaginal canal of the mole.

### (*f*) *The prostate in the female monkey.*

Prostatic rudiments which occur as para-urethral glands in the female rat can be activated by means of androgenic stimulation (Korenchevsky, 1937; Hamilton & Wolfe, 1937). These rudiments are believed to be homologous with the ventral lobes of the prostate of the male, and it is of interest that whereas all the prostatic lobes develop in female newborn rats when they have been markedly masculinized

as a result of androgenic stimulation, only the ventral lobes appear when the intensity of masculinization is slight (Greene *et al.* 1938*b*).

The urethral glands which occur in the human female are homologous with those prostatic tubules of the male which develop from the urethra above the level at which the Wolffian and Müllerian ducts enter the urogenital sinus (Johnson, 1920, 1922). The tubules in question are those of the middle (prespermatic) lobe, derived from the dorsal wall, those of the anterior lobe, derived from the ventral wall, and the more cranial tubules of the two lateral lobes, which apparently extend into this region (Lowsley, 1912, 1930).

Similar glands, although rare in *Macaca mulatta*, the rhesus macaque, sometimes occur in monkeys of the genus *Cercopithecus* (Sandys & Zuckerman, 1938), and they can be activated by means of androgenic stimulation (Zuckerman, 1938*c*). What is more significant, however, is that in the green monkey, *C. aethiops sabaeus*, glands of identical appearance occur as anterolateral offshoots of the part of the vagina immediately above the opening of the vagina. In one anomalous specimen, these glands formed a solid mass which was indistinguishable from prostatic tissue. This prostatic mass extended upwards and around the posterior surface of the vagina. A glandular mass indistinguishable from prostate was also found in a rhesus monkey, *Macaca mulatta*, and the common collecting duct of this gland passed into the dorsal wall of the vagina. Similar abnormally disposed prostatic tissue may occur in man. Thus, a prostatic acinus is in very close relation to the vagina of a human pseudo-hermaphrodite figured by Young (1937).

It may be safely assumed that the openings of these prostatic glands indicate their embryological origin. Since the prostate is undoubtedly a derivative of the urogenital sinus, these observations therefore suggest that at least the part of the vagina from which prostatic glands sprang in these abnormal cases must have been derived, not from the Müllerian ducts, but from the urogenital sinus.

(*g*) *Vaginal development in other mammalian species.*

The development of the vagina has been investigated by the usual histological methods in several species, but the results obtained are by no means uniform. Indeed, it is not only a case of agreeing with Baxter (1933*b*) that the components of the vagina may differ considerably in different orders, and even among the members of one order of Mammalia—it is even more a case of recognizing that the same method of study has in almost every instance led different competent observers to altogether different conclusions. In some animals, and by some observers, the vagina is regarded as purely Müllerian in origin. In others it is regarded as being formed of both Müllerian and Wolffian components, or of Müllerian and urogenital sinus components. In a few instances it has even been regarded as a product of all three.

The difficulty in answering the problem of the development of the vagina by usual embryological methods arises from the fact that where the Wolffian and Müllerian ducts make contact with the urogenital sinus, they are also generally in contact with each other. It becomes largely a matter of opinion as to how far

each of these ducts, or the urogenital sinus, contributes to the solid cord of cells which marks their termination. Langenbacher (1882), for example, regarded this cord as entirely Müllerian in origin in the rabbit. Baxter (1933*a*), on the other hand, is firmly convinced that both Wolffian and Müllerian cells are present in the terminal vaginal "knot" of this mammal.[1] His description of the cellular "bulbs" which form this knot, and of their replacement of degenerating Müllerian cells, is again practically identical with that given for other mammals (e.g. man), by other observers—with this very important exception, that some of these observers (e.g. Bloomfield & Frazer, 1927) regard the sino-vaginal bulbs as being Müllerian in origin, and yet others (e.g. Vilas, 1933; Koff, 1933) as derivatives of the urogenital sinus. Baxter's further description of the development of the vagina of the pig (1933*b*), and his discussion of the literature on the development of the vagina in ungulates generally, show quite as plainly how difficult it is to decide, by means of simple histological methods, the derivation of the cellular components of the distal part of the urogenital canal.

Perusal of the literature suggests, indeed, that while it may be impossible to accept the view that the formation of the vagina is a uniform developmental process in Eutherian mammals, it is even more difficult to accept, as final, conclusions about the origin of the epithelium of the vagina that are indicated by the usual embryological methods. On the other hand, evidence from other directions is, as shown above, almost incontrovertibly in favour of the view that the vaginal epithelium is either wholly or partly derived from the urogenital sinus. The stronger this evidence is, the weaker becomes not only the view that it is derived from the Müllerian ducts, but also the inference that Müllerian tissue responds to oestrogenic stimulation both by glandular (uterine) and squamous (vaginal) responses.

### (2) *The responses of the urogenital sinus*

#### (*a*) *Migration of sinus epithelium.*

The view that vaginal epithelium is derived from the urogenital sinus is entirely in accordance with the endocrinological evidence. Thus the response of the vagina of monkeys to oestrogenic stimulation is the same as, and can hardly be differentiated from, that of the vestibule, which is undoubtedly a vestige of the urogenital sinus. Similarly, the oestrogenic response of the "uterus masculinus" (or more correctly, vagina masculina) of many species of monkey is identical with that of the urethral epithelium, which, too, is derived from the urogenital sinus. These facts raise the further problem of deciding the upper limit of sinus involvement in the vagina.

The cervical line of transition of vaginal and uterine epithelium would suggest that this limit is the cervix (as embryological evidence suggests it is in the mouse), in the same way as the corresponding line of transition between rectal mucosa and

[1] The fact that the vagina of the rabbit is of compound origin is reflected in the complex character of the vaginal mucosa in this species (Carleton, 1931). In its lowest third it consists of stratified squamous cells, whereas in its middle third the epithelium is non-ciliated columnar, and in its upper third a mixture of ciliated and non-ciliated columnar. The non-ciliated columnar cells are phagocytic, and it is the lower stratified epithelium which reacts in a typical way to oestrogenic stimulation.

the epithelium of the anal canal marks the upper limit of the proctodaeum. This inference, however, is opposed by the fact that there is clear evidence that the Müllerian ducts in many mammals do take part in the formation of at least the upper part of the vagina.

An answer to this difficulty is the strong likelihood that in the normal ontogeny of many forms (the rabbit is presumably an exception), sinus epithelium from the lower part of the vagina ultimately replaces the Müllerian epithelium of the upper zone. Evidence in favour of this hypothesis comes from Baxter's study of the development of the vagina of the pig (1933*b*). At the end of foetal life the upper part of the vagina of this animal is lined by a single layer of columnar cells, while the lower part, to which Baxter believes the urogenital sinus contributes, is stratified. Transformation of the upper cylindrical epithelial lining into a stratified mucosa begins late in foetal life, and is not completed till sexual maturity. What is important here is that the transformation begins at the lower end of the columnar-cell lined segment, and slowly works upwards. This process strongly suggests the replacement of true Müllerian epithelium by caudal and different epithelial elements.

Further evidence of the participation of epithelium of the urogenital sinus in the formation of the upper regions of the utero-vaginal canal is given in the work of Dantchakoff and Raynaud. Thus Dantchakoff (1937*b*) writes of the sudden replacement of the typical uterine epithelium in the lower tapered part of the foetal guinea-pig uterus by cells identical with those of the urinary tract. Raynaud (1938*b*, 1939 *d*, *e*, *f*) describes an almost identical process in the mouse. His observations go even further and suggest that not only the epithelium of the utero-vaginal canal, but also that of the Wolffian ducts may be "replaced" by sinus epithelium.[1] Thus he found that the distal part of the Wolffian ducts of the normal newborn mouse is lined by epithelium different from typical Wolffian epithelium and identical with that of the urogenital sinus, especially with that of the dorsal wall of the sinus, where the characteristic clear sinus cell is most abundant.

These observations suggest that epithelium of the urogenital sinus tends to track away from the sinus along the paths formed by structures which obtain attachment to it. This view was originally put forward to explain the fact that stratified squamous metaplasia occurs in the ejaculatory ducts of rhesus monkeys which have experienced prolonged oestrogenic stimulation (Zuckerman, 1938*a*). It is not only supported by the observations referred to above, but also by the fact that cranially directed diverticula frequently occur in the prostatic urethra of monkeys (Zuckerman, 1938*a*). These diverticula may take the form of simple shallow pockets and blind tubular recesses, as well as common openings for the ejaculatory ducts and the "uterus masculinus" or for one ejaculatory duct and the "uterus masculinus". The latter condition is stated to occur in 8% of cases in man (Vintemberger, 1926). According to Bloomfield & Frazer (1927), the human

[1] It may be noted that some embryologists have put forward the view that the distal ends of the Wolffian ducts contribute to the formation of the lower end of the vagina in certain mammalian species. If this view is in fact correct, Raynaud's observation raises the very important question as to whether the cells which the Wolffian ducts contribute are in fact Wolffian duct epithelium or cells of sinus origin (see footnote p. 241).

Wolffian and Müllerian ducts remain separate in their entire extent, and open independently into the urogenital sinus. Consequently, it follows that the common opening of the ejaculatory ducts and "uterus masculinus" may be regarded as an evagination, or diverticulum, of the urogenital sinus, into which both the terminal part of the genital cord (vagina masculina) and the Wolffian ducts or duct (vas deferens) open.

All these observations suggest that the epithelium of the whole vagina, in most mammals that have been investigated, is derived, either from the start or by a secondary process of replacement, from that of the urogenital sinus. This conclusion can obviously be related to the fact that the vagina and uterus respond differently to oestrogenic stimulation. It can also be related to the fact that the coagulating and other lobes of the mouse prostate which, like the vagina, give the squamous response to such stimulation, take origin, not from Müllerian epithelium as was suggested by those who made the first observations on the sensitivity of the coagulating glands but, as Raynaud has shown, from the urogenital sinus itself.

(*b*) *Stratified squamous metaplasia.*

The observations which have been considered above bear out the suggestion (Zuckerman, 1936*a*, *b*) that epithelial metaplasia and stratification in the reproductive tract in response to oestrogenic stimulation, whatever its histochemical basis, may in general be regarded anatomically as a primary response of tissue in whose development oestrogen-sensitive sinus epithelium has played a part. Under normal conditions it would seem that stratified epithelium does not continue to invade those reproductive organs, whose original epithelium it had not completely replaced by the time of birth, partly because of the lack of a sufficient oestrogenic stimulus, and partly because of the counter-influence of the factors upon which the normal condition of the organs in question depends. In such organs the point where glandular and stratified squamous epithelium meet is an unstable zone of transition such, for example, as is found in the cervix uteri. When the oestrogenic stimulus becomes dominant, stratified epithelium gradually replaces the normal glandular mucosa, the change usually beginning in the region closest to the original urogenital sinus. When the stimulus weakens, the stratified epithelium recedes, and the mucosa returns to its normal condition.

While this hypothesis explains the general course of the metaplastic effects which oestrogenic stimulation induces in both the male and female reproductive tracts, it raises the further problem of how the metaplastic change is actually effected. An advancing wave of rapidly proliferating squamous sinus epithelium does sometimes replace an original less dominant Müllerian or Wolffian columnar epithelium. Such a process, according to Loeb *et al.* (1938), is the usual way in which the uterus of an oestrogenized mouse becomes covered by stratified squamous epithelium. It does not come, however, within the defined scope of the term "metaplasia". Adami & McCrae's (1914) authoritative definition of the term, as re-emphasized by Ham (1932) in Cowdry's *Special Cytology*, is as follows: "metaplasia is the postnatal production of specialized tissues from cells which

normally produce tissues of other orders, and is an adaptation on the part of the cells to altered environment....Metaplasia is not direct, but can be brought about only by a preliminary reversion to a vegetative type of cell or, where mother cells are present, by the development of cells modified by environment."

True metaplasia of this kind is possibly the process that has occurred in regions of the reproductive tract where isolated peninsular extensions of stratified squamous cells project into the lumen of a gland or duct; for these peninsular extensions could clearly represent the proliferation of de-differentiated columnar epithelium. On the other hand, such foci of "metaplasia", especially in regions close to the original urogenital sinus, might represent the proliferation, in the presence of an adequate stimulus, of dormant "squamous" cells derived originally from the sinus, or from a derivative of the sinus. In his general discussion of metaplasia, Karsner (1938) refers to such a possibility as follows: "what appears to be metaplasia may be due to inclusion of embryonal rudiments in the areas affected, which remnants may proliferate to form the new type of cells." A further possibility, in the case of the "metaplasia" we are discussing, is that the foci of abnormal proliferating cells may represent the distal ends of tracks of squamous epithelium, which had lost connexion with their source, but which originally were part of a general process of invasion.

The way the transformation takes place is so little understood that it is well worth while quoting in full the observations made by Loeb *et al.* (1938) in their recent study of the growth processes induced by oestrogenic hormones in the uterus of the mouse. They write as follows:

In one case we noticed as a first change two or three layers of loosely arranged cylindrical cells in which mitosis occurred; in another case a layer of cuboidal cells developed below the layer of cylindrical cells; in still other instances several layers of cuboidal cells formed beneath the cylindrical epithelium papillae, raising the latter up. Sometimes four or five layers of cuboidal cells were seen, and the upper cells, instead of keratinizing, underwent hyalinization, a process which is quite common in the cervix. The hyalinized cuboidal cells had a tendency to dissolve, as was evidenced by the formation of vacuoles. In the end such a vacuolization affected the whole cell, which was transformed into a soft colloid or gelatinous material. These vacuolization and solution processes were especially noticeable in the cuboidal hyaline cells which lay on top of the squamous epithelium; but they occurred, too, in the cuboidal hyaline cells which developed beneath the cylindrical cells and pushed the latter upwards. Hyaline material developed also in the cytoplasm of the cylindrical cells.

The epithelial cells which underwent the change into transitional and squamous epithelium apparently acquired thereby a greater mobility. This was manifested in two ways: (1) the squamous epithelium moved beneath the cylindrical epithelium where it adjoined the latter and raised it up from the underlying connective tissue; (2) where the squamous epithelium directly covered and thus came in immediate contact with relatively soft hyaline material which had been deposited in the mucosa, it moved downward as a connected row of cells into this gelatinous substance, partly traversing it. There is evidently inherent in the squamous epithelium a tendency to move, but under normal conditions extension into the underlying connective tissue is prevented by the firm consistency of the latter. The relatively soft hyaline material which develops in mice injected with large amounts of oestrogen does not offer the same degree of resistance, but on the contrary, acting as a foreign body, it may exert a motor stimulus on these cells.

The formation of transitional or squamous epithelium may take place not only in the surface epithelium, but also in the glands. In some cases it seems that the squamous surface epithelium grows downward into the glands; but in other instances low squamous epithelium may develop in the glands in places where cylindrical epithelium covers the surface of the uterus. Only rarely is it merely the gland duct which is lined with squamous epithelium; as a rule, the latter extends through a part or the whole of the body of the gland. Thus it may happen that one part of a gland fundus is lined with cylindrical and another part with squamous epithelium.

In uterine glands which have undergone this change the squamous epithelium may give origin to cuboidal nucleated, hyaline cells which fill the gland lumen. These glands then assume the characteristics of glands, or of precursors of glands, which are often seen in the cervix near the junction with the uterus. The desquamated hyaline cells may also, in certain cases, have a tendency to dissolve and this gives origin to colloid material which quite commonly distends these cystic uterine glands. There exist thus transition stages between the typical uterine cysts, lined with cylindrical or cuboidal epithelium and filled with a colloid gelatinous material, and the cysts of the uterine cervix filled with proliferated and desquamated hyaline cells, a condition related to and preceding the stages of keratinization.

By such processes of metaplasia (which in the male are undoubtedly best demonstrated at first in the region of the urethra, i.e. urogenital sinus), and by the subsequent proliferation of metaplastic areas, the transformation of the whole male genital tract of the mouse, as described by Burrows (1935*b*), may take place. It is also possible to explain, by means of the general hypothesis developed above, the metaplasia which was observed by Raynaud (1937*a*) in the upper urethra and in the base of the bladder of cats, and by Lacassagne (1935*b*) in the bladder of mice. The basis of this response would appear to be the presence in the neck of the bladder of cells both identical and continuous with the oestrogen-sensitive sinus epithelium (Raynaud, 1939*d*, *e*, *f*). The fact that there is a gradient of response (both in time and degree of change) between organs (see p. 235) is presumably explained by the fact that the various male accessory organs bud from the urogenital sinus at various distances from the most sensitive part of the sinus—which in most species appears to be the summit of the crista urethralis.

The existence of a corresponding gradient in the utero-vaginal canal of the female mouse is described as follows, from a slightly different point of view, by Suntzeff *et al.* (1938): "The nearer the tissue approaches, in its normal situation, the vaginal orifice, the greater is the tendency of the epithelium to grow in the form of squamous epithelium and the less its tendency to grow as cylindrical, gland-like epithelium...while in the vagina proper there is a tendency of the squamous epithelium to keratinize, in the cervix this tendency is more and more replaced by a tendency to hyalinize...and still farther up we may find instead of hyalinization a vacuolar degeneration of the upper cell layers."

It is not suggested that the kind of metaplasia described above is the only kind of abnormal epithelial change that can affect the reproductive tract. Squamous transformation of normal cylindrical epithelium occurs in many parts of the body as a result of irritation (e.g. in the gall bladder or the pelvis of the kidney when stones are present). It may also occur in response to friction over exposed parts

of a prolapsed uterus, and in this connexion it should be noted that Suntzeff *et al.* (1938) regard friction as an important factor in the localization of the stratified squamous response in the utero-vaginal canal of oestrogenized mice. Metaplasia, according to the terms of its definition, is an adaptation of cells to an altered environment. Friction represents a mechanical change, oestrogenization a chemical alteration in the environment of cells. Both, presumably, may operate in the reproductive tract.

(*c*) *Insensitivity of sinus derivatives.*

It seems clear that the squamous response to oestrogenic stimulation observed in the reproductive organs represents, from the anatomical point of view, a primary response of oestrogen-sensitive epithelium of the urogenital sinus.

Not all derivatives of the urogenital sinus, nor indeed the entire urethra, are, however, always sensitive to oestrogenic stimulation. In the mouse they appear to be, but in different species of monkey as well as in man, various derivatives of the sinus appear to be insensitive. Thus in the rhesus monkey the segment of the male urethra cranial to the openings of the Wolffian and Müllerian ducts, like the urethra of the female of the species, is generally insensitive, whereas all the urethra distal to these openings is sensitive. Similarly only the proximal parts of the prostatic collecting ducts are sensitive, whereas none of the epithelium of Cowper's glands is. In man, on the other hand, the only region of the urethra which is sensitive is the part immediately above and below the openings of the ejaculatory ducts, while the collecting ducts of the upper parts of the organ appear to be much more sensitive than they are in the rhesus monkey. On the other hand, Cowper's glands are insensitive, as they are in the rhesus monkey.

It is difficult to understand why different primates vary in the degree to which their urethral canals are sensitive to oestrogenic stimulation. There seems, however, to be some correlation between this variation and interspecific variation in the extent to which females of the species experience sexual-skin swelling (see below). It is equally difficult to understand how some structures which have developed as buds from oestrogen-sensitive sinus epithelium should later be unresponsive to oestrogenic stimulation (e.g. Cowper's glands).

It is, however, conceivable that the oestrogenic sensitivity of structures derived by budding from the sinus diminishes progressively with the degree of subdivision and elaboration of the original buds. Alternatively, it may be that the lack of response of the true prostatic glands or, for example, of the ducts and acini of Cowper's glands, is due to the later specialization of cells which primarily arose from oestrogen-sensitive epithelium. That one or both of these processes must have occurred is indicated by the fact that in the rhesus monkey the urethral openings of the insensitive prostatic glands, which presumably give the topographical relations of the embryonic prostatic tubules, are in fact placed almost entirely in the uppermost part of the region of the urethra which responds to oestrone (Zuckerman, 1936*a*), while Cowper's glands also undoubtedly develop from a region of the sinus that is sensitive to such stimulation.

A further possibility in the case of the prostatic ducts is that the primordial prostatic tubules may have been derived from a region of the urogenital sinus that is originally insensitive to oestrogenic stimulation. Sensitivity in this region and in the collecting ducts may have been attained later either by a change in the character of the original epithelium or by the replacement of that epithelium by more caudally disposed sensitive cells.

A third view is that the sensitivity of the whole sinus (and of some of the tubular structures which open into it) is a characteristic acquired after the glands to which the sinus gives rise have budded. Such a view seems less likely, however, than the one which implies that glandular organs derived from the urogenital sinus may become specialized, from the point of view of oestrogenic sensitivity, after they have budded.[1]

## IV. THE ECTODERM IN RELATION TO THE UROGENITAL SINUS

Thus far the analysis has been directed mainly at an examination of the view that organs with presumably the same embryological origin may give both glandular and squamous responses to oestrogenic stimulation. This view was in the first place suggested by the fact that the uterus gives the one, and the vagina the other kind of response, and by the fact that both organs are usually supposed to be Müllerian derivatives. But as has been shown, this embryological doctrine is highly questionable, for while the uterus is clearly a derivative of the Müllerian ducts, the vagina is almost certainly histogenetically related to the urogenital sinus. The proposition that has been tested thus falls to the ground, and so far as the Müllerian system is concerned the difference between the responses of the two organs could be made the basis of a more specific working hypothesis that oestrogenic stimulation evokes only a glandular response in organs that are solely derived from the Müllerian ducts.

The further problem that tissues which are usually presumed to have widely different origins (i.e. Wolffian and entodermal derivatives) may nevertheless exhibit the same type of squamous response to oestrogenic stimulation is also clarified by the foregoing analysis. In this case it has been demonstrated that the common squamous response may be related anatomically to the fact that the epithelium of the urogenital sinus has played a part in the development of all the internal organs concerned, e.g. vagina, prostate, urethra. The squamous response appears, however, to be less specific than the glandular, for it is exhibited not only by derivatives of the urogenital sinus, but also by a variety of ectodermal tissues (e.g. skin, gums). It becomes necessary, therefore, to enquire into the anatomical relationship of the entodermal (urogenital sinus) squamous response to the ectodermal (skin) squamous response.

### (1) *The response of skin to oestrogenic stimulation*

As noted above, the genital and circumgenital skin in many species of monkey is sensitive to oestrogenic stimulation. In the normal mature female of the species

[1] The urogenital sinus has been found to be sensitive to oestrogens not only in foetal and newborn rodents, but also in the newborn pouch opossum (Burns, 1939).

this sensitivity shows itself, during the follicular phase of the menstrual cycle, not only by reddening and swelling of the reactive area, but also by proliferation of the epidermis. Desquamation also occurs. The corresponding region in the male of the species does not undergo any changes under normal conditions, but injection of oestrogenic hormone shows that it is equally sensitive.

As has also been pointed out, the limits of the reactive zone vary in different species and at different times. Thus the change is most marked in animals like baboons (*Papio* spp.), moderately developed in a species like the rhesus monkey (*Macaca mulatta*), and not apparent macroscopically in a species like the green monkey (*Cercopithecus aethiops sabaeus*). An attempt to analyse the relative extent of the change in different parts of the body has been made in the latter two species.

It was shown in a previous study (Zuckerman *et al.* 1938) that in its maturation the sexual skin of the rhesus monkey passes through three main types of response: first, the limitation of oedema to a circumgenital zone; second, the peripheral extension of the zone of oedema and its disappearance from the central circumgenital area; and third, the general disappearance of subcutaneous oedema, and the final restriction of the main zone of coloration to the perineal region of the body. These three phases can be induced artifically, in both male and female immature rhesus monkeys, by the continued injection of oestrogenic hormone.

The extent of the reactive region, both in normal mature females and in experimental animals of either sex that are being subjected to prolonged intensive oestrogenic stimulation, sometimes increases in a very remarkable way. Thus:

(*a*) The supraorbital ridges and the region of the nasion may swell, and remain swollen over long periods.

(*b*) Occasionally parts of the main sexual-skin region other than the central zone become oedematous in mature animals. When it occurs, this change is usually confined to the periphery of the main sexual-skin region.

(*c*) Oedema of the skin of the posterior aspects of the legs, as far down as the tendo Achilles, is also occasionally observed in fully mature rhesus monkeys.

(*d*) The skin of the whole back may be thrown into a series of thick oedematous folds. When this occurs, the skin of the supraorbital ridges is also usually thickened and swollen, and overhangs the eye sockets, while the skin of the temporal fossae is thrown into obliquely directed folds. The swellings of the back may extend forwards as far as the lateral lines of the abdomen, and the navel may be everted. The skin of the lower abdominal wall may also become somewhat swollen. When swelling of this kind is resorbed, the skin of the back is usually, for a considerable time, broken up into a tesselated arrangement of thick, grey, irregularly formed plaques.

Like the normal reactive zone, proliferation of the epidermis occurs in these extragenital zones of swelling.

In a series of observations of which details will be published elsewhere (Aykroyd & Zuckerman), it was found that the circumgenital skin changes may be regarded as part of the same oestrogenic process of stratification that takes place in the vagina of the female monkey and in the urethra of the male. These observa-

tions were made on normal immature rhesus monkeys and on immature rhesus monkeys that had been injected with oestrone. Measurements were made of the thickness of the stratified epithelium in thirty-six pieces of skin taken in each animal from arbitrarily chosen points on the mid-ventral, mid-dorsal and lateral lines of the trunk and head, and from the medial-adductor and dorsal lines of the inferior extremity, and also of the thickness of stratified epithelium in the reproductive organs. It was found that the epidermis in the genital and central sexual-skin area was thicker in the injected than in the control animals, and that the difference in thickness became increasingly greater towards the vulva and vagina. A similar but less pronounced change was observed in the nape of the neck, over the crown of the head, and to a lesser extent over the upper part of the nose. There appeared to be no change in other regions of the body. Estimates were also made of the degree of mitotic activity in each specimen of skin, and these showed that the rate of mitosis was far higher in the responsive regions of skin than elsewhere, the number of dividing cells increasing with the thickness of the epidermis.

A more limited number of observations were made on the green monkey (*Cercopithecus aethiops sabaeus*), but they were sufficient to show that the thickness of the vulval skin in front of the vaginal opening increases considerably during the course of oestrogenic stimulation, and that the increase in thickness is greatest over the inner aspects of the labia minora. There is very little response more peripherally, a fact that can be correlated with the absence of a sexual skin in this species.

Whether or not they could be distinguished histochemically, there is at present no obvious criterion by which to differentiate between the stratified squamous oestrogenic response of the vagina, that of the vestibule, and that of skin lying more peripherally to the vestibule. These observations therefore suggest that the external circumgenital reactive area may be regarded as the peripheral part of a total oestrogen-sensitive epithelial zone. The most sensitive part of this zone in the female is the vagina, and, to some extent, the response, under normal conditions of stimulation, diminishes progressively the farther one proceeds from the very sensitive central area. There is, in fact, a gradient of epithelial sensitivity in this region, the peak of the gradient lying centrally, in the vagina, and the fall of the gradient being correlated with the extent of the sexual-skin area. Thus in the rhesus monkey the fall is gradual, whereas in the green monkey, which has no sexual skin, only the skin of the vestibule and immediately adjacent areas seems to respond. In both species, however, the entodermal (urogenital sinus) squamous response grades into the ectodermal (genital and circumgenital skin) squamous response, and no sharp distinction can be made between the two.

### (2) *The relation of urethral changes in the male monkey to sexual-skin changes in the female*

There appears to be a relation between this finding and the variations which occur in the extent to which the urethral epithelium in male monkeys undergoes stratified squamous metaplasia as a result of oestrogenic stimulation (p. 237).

The extent of the change in different species correlates fairly closely with the degree to which the external genitalia and circumgenital skin of the corresponding females are sensitive to oestrogenic stimulation. Thus the pronounced changes occurring in the urethra of the male rhesus monkey, common macaque, and baboon, are reflexions of the marked changes that occur in the sexual skin of the females of these species. Again, the apparent absence of marked changes in the urethra of the male langur, the common marmoset, the capuchin, and man is reflected in the absence of any obvious oestrogenic changes in the circumgenital skin of the females of these species. The experimental data available do not, however, allow any wider generalization than that in species in which stratification of the male urethral epithelium has not yet been observed, the corresponding females have no external sexual skin; whereas in species in which the female has a sexual skin, stratification of the male urethral epithelium occurs. This generalization does not cover those exceptional cases where extensive urethral stratification occurs in the males of species in which the female does not exhibit any macroscopic sexual-skin changes.

The part of the male urethra that was found to be sensitive in all species of primate that have been tested on the one hand corresponds to the region of Müllers tubercle in the embryo, and on the other to the opening of the vagina in the fully formed female. Its great sensitivity can thus be related to the corresponding sensitivity of an homologous region in the female. Similarly, the fact that the part of the primate male urethra above the level of the utricular mouth is generally insensitive to oestrogenic stimulation, may be related to the finding that the urethra of female monkeys, with which this part of the male urethra is homologous, is also, as a rule, insensitive. It may also be related to the likelihood that during its development this part of the male urethra incorporates epithelial elements from the Wolffian ducts (Frazer, 1935). It may be noted again in this connexion that, in monkeys, the urethral openings of the prostatic collecting ducts, which presumably give the topographical relations of the embryonic prostatic tubules, are usually placed immediately below and above the mouth of the "uterus masculinus". The fact that some of the glands arise from an insensitive part of the urogenital sinus is perhaps an added explanation for the insensitivity of the prostatic glands themselves (see p. 252).

### (3) *The mammary ridges*

Thus far we have considered the gradient of oestrogenic response in only genital and circumgenital skin. Whether or not this gradient varies according to the stage of maturation of the sexual skin remains to be determined. Further problems that await investigation are (*a*) the possibility that a gradient exists at the cranial end of the body, in the skin of the head and in the epithelium of the stomadaeum, and (*b*) the relationship of the circumgenital skin gradient to the skin changes that occur under abnormal conditions over the trunk and legs, and of the latter changes to the changes that occur in the head.

Further evidence of the occurrence of a gradient of oestrogenic sensitivity in ectodermal tissue can be found in the behaviour of the mammary ridges of the

embryo. These reach on each side from the base of the hindlimb bud to the base of the forelimb bud. The number of separate glands and nipples that develop from them varies in different mammals. In some, nipples occur at regular intervals along the mammary ridges all the way from the inguinal region to the axilla. In others, the mammary glands are restricted to the pectoral region, and in others again to the inguinal region. In the Cetacea, for example, the nipples are situated in slits on either side of the genital groove (vulva).

In the female rat and mouse nipples are present in both the pectoral and inguinal regions. They cannot be recognized macroscopically in the males of these species. Nipples develop, however, in feminized genetic males (Greene *et al.* 1938*a*; Raynaud, 1939*a*), and Raynaud observes that it is the most caudal pair that is most frequently and best developed. This finding can be related to our observation that the most sensitive part of the sexual-skin area is the region closest to the central zone of the external genitalia.

### (4) *The development of the urogenital sinus*

Whatever be the interrelations of the different ectodermal fields of oestrogenic sensitivity, it is clear that the vagina and male urethra on the one hand, and the genital and circumgenital skin on the other, may be regarded as together constituting a single zone of sensitivity. According to accepted embryological doctrine, in which a sharp distinction is made between the different germ layers, the epithelium of this field of activity is derived on the one hand from ectoderm (skin) and on the other from entoderm (urogenital sinus). There is, however, an alternative view, which is examined below, that ectoderm also plays a part in the formation of the distal part of the urogenital sinus (see Frazer, 1931). This view certainly finds indirect support in the endocrinological evidence. It also harmonizes with the fact that the epithelium of the anal canal, which is derived from the proctodaeal invagination of ectoderm, closely resembles that of the vagina. This resemblance was remarkably clear in an abnormal rhesus monkey that possessed a recto-vaginal fistula (Sandys & Zuckerman, 1938), and a rectum which opened to the exterior by a normal anal canal. The fistula led into the posterior fornix. The epithelium of the fistula, and of the rectum over and distal to the cervix uteri, was stratified squamous, and was therefore presumably related to the ectodermal proctodaeum (the epithelium of the lower rectum in the rhesus monkey is normally glandular). Moreover, the epithelia of the "rectum", the recto-vaginal fistula and the vagina were continuous, and in appearance identical. There was as much shedding of superficial cornified epithelium in the lower rectum as in the vagina. It would seem reasonable to suppose that the three epithelia were related histogenetically. And since there can be no question that the proctodaeum is of ectodermal origin, the presumption in this case would be that the vagina was histogenetically related to the ectoderm.

That ectoderm contributes to the formation of the vagina in the mole is indisputable (p. 242). Furthermore, recent studies by Barnstein & Mossman (1938), on the red squirrel (*Tamiasciurus hudsonicus*), and by Siddiqi (1937) on the ground

squirrel (*Citellus tridecemlineatus*), provide the clearest evidence that the penile urethra and its bulbo-urethral and bulbar glands, which are generally believed to be of purely entodermal (urogenital sinus) origin, are in fact wholly, or almost wholly, ectodermal structures. Since the developmental changes described by these authors are closely similar to those described in other mammalian species, it is of interest to consider the details of the morphogenetic process so as to discover why one group of investigators regard the penile urethra as ectodermal, and another as entodermal in origin.

The term urogenital sinus applies, strictly speaking, only to that part of the ventral division of the entodermal cloaca which lies caudal to the attachment of the Wolffian and Müllerian ducts. It is separated from a very shallow ectodermal cloaca (which is bounded laterally by the genital folds, and in front by the genital tubercle) by the cloacal membrane. This latter structure is lined on its inner aspect by entoderm, and on its outer by ectoderm; in practice, however, it is usually described as consisting of "a mass of epithelial cells", in which it is almost impossible to distinguish its thickened ectodermal and entodermal components (e.g. Lewis, 1912).[1] Felix (1912) describes the ectodermal contribution to the membrane as being the richer, while most authorities are agreed that the membrane incorporates part of the primitive streak (e.g. Keibal, 1910; Pohlman, 1911; Lewis, 1912; Keith, 1923; Frazer, 1931).

As the genital tubercle develops into the phallus, the distal part of the urogenital sinus (pars phallica) is usually described as being drawn-out in it. It is not, however, prolonged as a cavity with an open lumen, but as a "potential cleft" in the form of a supposedly double epithelial septum. This septum, to which, among others, the terms "cloacal septum" or "urethral plate" have been applied, is in every case continuous with the ectoderm on the under-surface of the genital tubercle. Some authors (e.g. Felix, 1912) believe that the whole of the pars phallica becomes occluded and transformed into a more or less solid septum.

The further course of development of the urogenital sinus is different in the two sexes. In the human female, according to Frazer (1931), the pars pelvina of the sinus disappears owing to its invagination by the sino-vaginal bulbs, while the pars phallica opens to the exterior (presumably as a result of the breakdown of the ventral part of the cloacal membrane), and forms the vestibule. Koff (1933) agrees with this description, but Felix (1912) does not refer to the invagination, and according to him the destruction of the urogenital membrane (ventral part of cloacal membrane), along its whole length, opens the pars phallica and pars pelvina, through the orificium urogenitale primitivum, to the exterior. With relatively little change, this opening becomes the vulval opening, the labia minora arising both from the margins of its ventral obliterated part (the "urethral groove") and from the margins of its dorsal part, which remains permanently open. According to Felix the

[1] If the cloaca is regarded as the intermediate zone between external skin and urogenital sinus, it is of interest that the reaction of the lizard cloaca to oestrogenic stimulation is the same as that of the mammalian vagina (Dantchakoff, 1938 *b*, *c*). The cloaca in these animals is of a very simple kind, and both ectodermal and endodermal elements play a part in its formation. Throughout its extent it undergoes marked squamous metaplasia in response to oestrogenic hormone.

vestibule is formed by both the pars phallica and pars pelvina of the urogenital sinus.

As in the female, the pars phallica of the male, through a breakdown of the urogenital membrane, first opens to the exterior. The opening is then closed, by fusion of the genital folds, from behind forwards, and the urogenital sinus is thus carried forwards to the tip of the developing phallus.[1] Descriptions vary, but it is usually suggested that the more proximal part of the pars phallica has a lumen, and that the "potential cleft", referred to above, exists only in the very distal part of the phallus. In the glans this "potential cleft" (which is the "cloacal septum" or "urethral plate") also breaks down and opens to the surface, and then fuses again, from behind forwards, to carry the meatus to the tip of the phallus.

The problem that has to be considered here is whether the entire urogenital sinus is laid down from the beginning, as Felix maintains, as an entodermal structure, or whether the pars phallica, especially the part which forms a solid urethral plate, is a later ectodermal derivative.

It is perfectly obvious that a process by which the penile urethra is formed through fusion of the genital folds makes it possible that this part of the urethra incorporates ectodermal elements from the ectodermal cloaca. This very real possibility acquires considerable force in view of Barnstein & Mossman's clear demonstration that the urethral plate in the squirrel is not a distal extension of the pars phallica of the sinus, but a derivative of the ectoderm of the under-surface of the phallus. The plate, from which Cowper's glands are derived, separates from the surface ectoderm, and is canalized from behind forwards to form the whole of the penile urethra. Siddiqi's study of the ground squirrel also makes it perfectly clear that in this species the distal part of the urogenital sinus is represented by a solid cord, to which a mass of cells, derived from the ectodermal cloaca, becomes opposed. This ectodermal mass of cells canalizes, and later the hinder entodermal urethra opens into it. From this ectodermal ingrowth there are finally formed the bulbar gland, the bulbo-urethral glands, the penile duct, and the part of the penile urethra distal to the opening of the penile duct. Comparison of this process of development with the process of urethral development as normally described for man, led Siddiqi to the view that "the proximal two-thirds of the mucosa of the dorsal part of the human penile urethra is entodermal in origin, while the bulb of the urethra, the floor of the proximal two-thirds, and the whole of the terminal third of the penile urethra are ectodermal in origin".

Felix's authoritative account of the development of the urogenital sinus appeared in that standard work of reference, Keibal & Mall's *Human Embryology*. Felix had not, however, worked on the problem himself. In presenting their own observations, Barnstein & Mossman point out, with obvious justification, that few embryologists have been prepared to dispute Felix's vigorous statement that the whole of the urogenital sinus, from the time it is first laid down until its transformation into

[1] It may be noted that Felix believes that the ostium urogenitale does not fuse from behind forwards, but that it is "passively" carried forward to the base of the glans, by "active" growth in more proximal regions of the penis (the region of the pars pelvina).

definitive organs, is an entodermal structure. This hesitation to tilt with authority has obscured the fact that there must always be considerable difficulty in deciding the cellular origin of the urethral plate. Thus, although it is "continuous with the ectoderm of the tubercle" in the white mouse, Hall (1936) nevertheless subscribes to Felix's view that it is of entodermal origin, and that it is formed by the fusion of the lateral walls of the pars phallica. This process of fusion does not, however, appear to have been observed, whereas it is obvious that "the extension of the cloacal cavity in the tubercle", which Hall regards as supporting Felix's opinion, as readily supports the more recent view of Barnstein & Mossman. The same difficulty applies to the problem of the formation of the urogenital canal in marsupials. The form of the urethral plate appears to be the same in these animals as it is in Eutherian mammals, and it is also "continuous with the ectoderm of the tubercle". Yet like Felix, Buchanan & Fraser (1918) regard it as formed entirely by the compression, and subsequent fusion, of the lateral walls of the most ventral and distal part of the urogenital sinus. From the text-figures provided, however, it seems as likely that the walls of this part of the sinus were in fact ectodermal ingrowths. Furthermore, the union of the entodermal and ectodermal cloacae after the breakdown of the cloacal membrane provides an obvious opportunity in marsupials for the infiltration of ectoderm into the tissues of the distal part of the urogenital tract.

It is perfectly plain that in making his choice between the embryological view that the urethral plate is an ectodermal structure, and the view that it is derived from the entoderm, the student interested in the reactions of tissues to oestrogenic stimulation can be confident that the criteria which decide this issue for the embryologist are no less arbitrary and subjective than those which operate in embryological discussions of the origin of the vagina. It is also clear that the embryological evidence already available leaves little doubt that at least the distal part of the urogenital sinus is histogenetically related to the ectoderm. Speculation about the extent to which the latter plays a part in the formation of the sinus is, however, idle, but it is not without significance that the floor of the urogenital sinus is the ventral part of the cloacal membrane. This membrane, as noted above, incorporates in its development the primitive streak, a structure which is homologous with the blastopore of lower amniota and anamnia. Both the primitive streak and blastopore are regions of intense proliferative activity, and both are the main focal points in the early organization of the developing organism. It is in this region of the early embryo that cells stream in from the surface to deeper structures. Although these processes relate to the first stages of embryonic life, it is therefore necessary to bear in mind both the fact that the region which in the mature organism represents the cloacal zone of the embryo is a region which in an earlier phase of its history was the centre of the formative movements of the body, and the possibility that its potency and plasticity are not completely lost in later stages of development. It may be, therefore, that there is as much a tendency for the distal ectodermal components of the urogenital sinus to impress their character on more proximal entodermal tissue, as there is for sinus epithelium to invade

structures which come into contact with it. One further connexion between the blastopore and the cloacal region which may be noted at this point is the fact that the organizing substance of the blastopore region is in all likelihood related chemically to the oestrogens (Waddington & Needham, 1935; Needham, 1936), and that it is in the urogenital sinus that the latter class of hormones finds its most sensitive zone of response.

Whatever be the further significance of these various considerations, it is plain that the participation of ectoderm in the formation of the distal part of the sinus can be related anatomically to the single gradient of oestrogenic response that is constituted by the reactions of urogenital sinus derivatives and circumgenital skin.

## V. CONCLUSION

The facts that have been brought together in this paper show quite clearly that although there are sharp developmental distinctions between various urogenital tissues that respond to oestrogenic stimulation, the embryological components of the urogenital system intermix, during development, at their points of contact. Thus the caudal ends of the mesodermal Müllerian and Wolffian ducts are invaded by epithelium from the primarily entodermal urogenital sinus, and the caudal part of the sinus, in turn, is infiltrated with ectodermal cells from the cloacal region. This conclusion conflicts with older embryological views.

There is this, however, that can be taken over from more conventional embryological teaching. Specific types of response to oestrogenic hormone do appear to belong to tissues that are derived from distinct embryological sources. Thus in monkeys the epithelium of the Fallopian tubes and corpus uteri, which apparently develops solely from Müllerian epithelium, responds altogether differently from the epithelium of the urogenital sinus. This fact suggests strongly that where the typical proliferative reaction of an organ like the uterus alters as a result of abnormal oestrogenic stimulation in the direction of the sinus response, a possible cause of the change, regarded anatomically, is an increased infiltration by epithelial elements whose normal behaviour it is to respond in that "abnormal" way. The vagina, although doubtlessly laid down as a Müllerian structure, is in its definitive condition almost certainly lined by epithelium ultimately derived from the urogenital sinus. The region where its mucosa meets that of the uterus is an unstable zone of transition, and an excess of oestrogenic stimulation leads to the proliferation of the vaginal type of epithelium, first in the cervix, and then, in some mammalian types, throughout the uterus. This reaction is no different from the corresponding one which the same stimulus would induce, for example, in the distal part of the ejaculatory ducts, which belong to the Wolffian system, for all points of contact of urogenital sinus epithelium and of epithelium belonging to the Wolffian and Müllerian systems may be regarded as zones of potential epithelial change. As has already been pointed out, the fact that progressive "metaplasia" does not occur in these organs under normal conditions indicates that the stimulus necessary for the continued proliferation and encroachment of stratified squamous epithelium

is usually lacking. Presumably, too, the fact that it is not the normal glandular uterine epithelium which spreads to the vagina to some extent supports the view, emphasized in a different connexion by Loeb *et al.* (1938), and established by Marrian & Parkes (1930), that the sensitivity of the endometrium to oestrogens, as denoted by proliferative activity, is weaker than that of the vaginal mucosa. On the other hand, the original "glandular" character of a metaplastic squamous area will reassert itself more and more as the strength of the oestrogenic stimulus wanes.

These considerations are not advanced as any hard and fast rule. They are only the basis of a working hypothesis that stratification in the reproductive tract in response to oestrogenic stimulation may in general be regarded, from the anatomical point of view, as a primary response of tissue in whose development sinus epithelium, either directly or indirectly, has played a part. And it must again be emphasized that stratification of epithelia which are not normally stratified does not necessarily imply the occurrence of "metaplasia" in the true sense of the term. Thus the stratification which occurs in the "uterus masculinus" and urethra of male monkeys as a result of oestrogenic stimulation can no more be described as a metaplastic process than could the stratification which a similar stimulus induces in the involuted vagina and vestibule of a spayed female monkey.

It should also be pointed out again that many tissues that are derived from apparently sensitive regions of the urogenital sinus are themselves insensitive to oestrogens. This indicates that histogenetically related tissues may specialize differently. Such specialization is also shown, in the case of ectodermal structures, by the fact that both feathers and mammary glands respond to oestrogens differently from other ectodermal derivatives. The possibility of specialized reactions is also indicated by the vaginal mucification that occurs under certain conditions of endocrine stimulation, although whether this process is metaplasia, or whether it represents the proliferation of dormant "mucifying epithelium" (Freud, 1937) cannot as yet be determined.

Wood (1936) has remarked that "a broad theory of metaplasia by breaking down all histological boundaries, really explains nothing, but merely avoids the problem". While metaplasia may, therefore, occur in the reproductive tract, the facts that have been brought forward in this article are a clear warning against the too-ready acceptance of the belief that the process has taken place wherever squamous epithelium is abnormally found in the urogenital tract as a result of oestrogenic stimulation. Furthermore, besides indicating what is plainly a valuable endocrinological approach to embryological problems, an approach that can avoid certain disputes over histological interpretation, the analysis followed also bears on problems of human pathology. Thus the fact that adeno-carcinoma is the usual type of cancer which affects the body of the uterus, whereas squamous carcinoma is the typical cervical cancer, is obviously related to the degree of involvement of squamous epithelium in the development of these two regions of the uterus. Furthermore, the fact that squamous cancer is extremely rare in the body of the uterus whereas it occurs relatively frequently in the uncommon disease of cancer of Bartholin's

glands (Rabson & Meeker, 1938), may also be associated with the closer developmental relationship of the latter organs to the urogenital sinus. It is also of interest that while primary carcinoma may occasionally occur anywhere in an otherwise normal prostate, it most commonly starts in the posterior lobe (Ferguson, 1932), the only lobe of the prostate that is entirely derived from the sensitive region of the urethra immediately below the openings of the Wolffian and Müllerian ducts. And as a last instance of the pathological bearing of the issues that have been raised, it may be noted that the very oestrogen-sensitive fibro-muscular tissue which is derived from the intermediate cell mass and genital cord is frequently the site of fibromyomatous growths, which, it has been argued (Witherspoon, 1935, 1936), are themselves the product of abnormal oestrogenic stimulation.

The analysis that has been pursued in this paper, by concentrating on developmental relationships, has thus succeeded in reaching a working hypothesis which appears to relate many, if not most, of the known proliferative effects of oestrogenic stimulation. The analysis permitted by the available facts cannot, however, as yet extend beyond the scope of Burrow's definition of the field of oestrogenic sensitivity (see p. 240). His statement indicates a functional aspect of the problem. Its more complicated anatomical aspect has been emphasized here. Many of the anatomical problems that require investigation are obvious. From the developmental point of view they apply as much to the epithelial interrelations of the stomadaeum and foregut, as they do to the epithelial relationships of the caudal end of the body.

## VI. SUMMARY

1. Oestrogens stimulate growth not only of the epithelial and fibro-muscular tissues of the male and female urogenital tracts, but also of certain non-reproductive tissues (e.g. skin, nasal mucosa, gums). Under normal physiological conditions oestrogenic sensitivity manifests itself histologically both in the orderly proliferation of cylindrical secretory cells (for convenience this response is referred to as "glandular"), and in the proliferation, cornification and desquamation of stratified squamous epithelium (for convenience called "squamous response").

2. In the main the glandular response is confined to tissues (e.g. uterus, hydatids of Morgagni) which are undoubtedly derived from the Müllerian duct. The squamous response, on the other hand, occurs in tissues which are stated to be derived from the Müllerian and Wolffian ducts, the urogenital sinus (entoderm) and the skin (ectoderm).

3. Presumed Müllerian tissues (e.g. uterus and vagina) are thus capable of both the glandular and squamous types of response. Examination of the embryological evidence on the development of the vagina in various species, and of evidence derived from the study of natural and experimental intersexuality, suggests, however, that the vaginal epithelium is ultimately derived from the urogenital sinus. It is likely that in cases where the Müllerian ducts contribute to the development of the upper part of the vagina, the Müllerian epithelium is as a rule finally replaced by sinus epithelium.

4. It is suggested that true Müllerian tissue responds to oestrogens by glandular proliferation, and that epithelial metaplasia and stratification in the reproductive tract in response to oestrogenic stimulation, whatever be its histochemical basis, may in general be regarded anatomically as a primary response of tissue in whose development oestrogen-sensitive sinus epithelium has either played a direct or an indirect part.

5. The process of metaplasia whereby the squamous response spreads to parts of the urogenital tract in which it does not normally appear is examined. The points at which the glandular epithelium of organs opening into what was originally the urogenital sinus meets squamous epithelium derived from the sinus are unstable zones of transition, and even in normal circumstances there may be a tendency for stratified squamous epithelium to replace epithelium which is normally cylindrical (in the same way as the originally Müllerian vaginal epithelium appears to be replaced during development by cells from the urogenital sinus). This tendency is increased under the influence of oestrogenic stimulation.

6. Derivatives of the oestrogen-sensitive sinus may themselves be insensitive.

7. The changes which oestrogens produce in the genital and circumgenital skin of monkeys may be regarded as the peripheral part of a total oestrogen-sensitive epithelial zone. The most sensitive part of this zone in the female is the vagina, and to some extent, the response, under normal conditions, diminishes progressively the further one proceeds from the very sensitive central area.

8. In species of monkey in which the female has a sexual skin, oestrogenic stimulation causes stratified squamous proliferation of the male urethral epithelium. In male monkeys in which stratified proliferation of the urethral epithelium has not been observed, the corresponding females have no external sexual skin.

9. Ectodermal elements appear to be involved in the development of the caudal part of the urogenital sinus.

10. It is therefore concluded that although there are developmental distinctions between various urogenital tissues that respond to oestrogenic stimulation, the embryological components of the urogenital system intermix, during development, at their points of contact. Thus the caudal ends of the mesodermal Müllerian and Wolffian ducts are invaded by epithelium from the primarily entodermal urogenital sinus, and the caudal part of the sinus, in turn, is infiltrated with ectodermal cells from the cloacal region. This represents a morphogenetic reflexion of the fact that the oestrogenic responses of the circumgenital and genital skin grade, without any sharp interruption, in the female into those of the vagina, and in the male into those of the urethra, and that the responses of these two organs in turn may extend into organs which open into them.

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## ADDENDUM

It was stated on p. 235 that the metaplastic effects of oestrogens in the reproductive organs of rats are less marked than in mice. A recent paper by E. M. Hume *et al.* (*J. Path. Bact.* 1939, **49**, 291) shows, however, that if oestrogenic stimulation in rats is combined with partial deprivation of vitamin A, metaplastic changes in the urogenital system become much more pronounced and extensive. Under these conditions metaplastic changes also occur in the thyroid, trachea and intrapulmonary bronchi, "but these changes occurred in about the same degree and with much the same frequency in the groups on – A diet alone as with additional administration of oestrogens". Nevertheless, their occurrence under these conditions would seem to provide a useful technique for studying the epithelial relations of the cranial end of the body (see pp. 256 and 263).

It was also stated on p. 235 that histological observations on the effects of oestrogens on the nasal mucosa have not been published. S. Soskin & L. B. Bernheimer (*Proc. Soc. exp. Biol., N.Y.*, 1939, **42**, 223), in another very recent paper, imply that the effects are not proliferative, but are mainly, if not entirely, hyperaemic.

Other noteworthy observations on the effects of oestrogens reported during the period this paper was going through the press were as follows: R. Tislowitz (*Anat. Rec.* 1939, **75**, 265) has provided a clear demonstration of the growth induced in the fibro-muscular stroma of the prostate and seminal vesicles of mice (see p. 235). S. R. M. Reynolds & F. I. Foster (*Amer. J. Physiol.* 1939, **126**, 606, and *J. clin. Invest.* 1939, **18**, 649) have published further data dealing with the question of the generalized vascular effects of oestrogens (see p. 232). A striking finding, which bears more closely on the question of the histogenesis of tissues sensitive to oestrogens, is that the pouch as well as the mammary glands of the marsupial *Trichosurus vulpecula* grows under the influence of oestrogenic stimulation (A. Bolliger & A. Carrodus, *Nature, Lond.*, 1939, **144**, 1049). The pouch muscles hypertrophy, while growth of the pouch skin is associated with the secretion of a deep orange pigment that stains the hairs of the region.

The homology of the "primary-primordia" of the mammary glands of marsupials with the "milk-streaks"—the precursors of the milk-lines—of foetal Eutherian mammals was established by E. Bresslau (*The Mammary Apparatus of the Mammalia*, 1920. London: Methuen). The sensitivity of the marsupial mammary gland to oestrogenic stimulation thus corresponds to that of the Eutherian mammary gland. Two views are possible about the sensitivity of the pouch. The first follows from Bresslau's belief that the pouch is formed during development by the confluence of several marsupial pockets, each of which contains a nipple. The contiguous walls of the separate pockets flatten out, while the outer walls of the most peripheral pockets remain as the wall of the definitive pouch. According to this analysis the marsupial pouch is a derivative of the mammary apparatus, with which its sensitivity to oestrogens could therefore be associated.

The alternative view is that the marsupial pouch is the homologue of the scrotum (D. Berry Hart, *J. Anat. Physiol.* 1909, **44**, 4). Berry Hart points out that rudimentary pouch folds occur in some male marsupials, but these he regards as "merely the folds after the scrotum has separated from its epidermis bed". He also holds that the genital skin zone in primates (e.g. the labia majora and the scrotum) is phylogenetically related to the suprapubic region in which the marsupial scrotum or pouch develops, and he emphasizes a developmental connexion between the caudal part, and what would seem to be the origin, of the whole mammary line and the genital zone. If these suggested relationships are true, the very interesting conclusion follows that the oestrogenic sensitivity of both the marsupial pouch and the whole mammary apparatus are linked developmentally with that of the circumgenetical skin.

# LES PIGMENTS DES INVERTÉBRÉS
## (À L'EXCEPTION DES PIGMENTS RESPIRATOIRES)

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(*Received* 28 *September* 1939)

TABLE DES MATIÈRES



## I. INTRODUCTION

Le but de cet article est de décrire les progrès récents de la chimie des pigments des Invertébrés et les notions biologiques nouvelles qui en résultent. Nous n'avons pas voulu énumérer tous les pigments décrits plus ou moins exactement depuis plus de cent ans. Nous décrirons avec plus de détails certaines matières nouvelles, pour lesquelles il n'existe pas encore de revue d'ensemble (quinones, pigments pyrroliques, ptérines). Pour des pigments bien connus, comme les caroténoïdes et les flavines, nous nous contentons de compléter des revues anciennes.

Nous avons exclu de cette revue les pigments respiratoires qui ont fait le sujet de beaucoup d'articles de revue (Barcroft, 1925; Redfield, 1933; Bigwood, 1935; Florkin, 1934 *a*, 1934 *b*; Roche, 1936).

Les ouvrages de Fürth (1903), Samuely (1911), Verne (1926), Bergmann (1933), Fischer & Orth (1937) nous ont beaucoup servi pour la préparation de cet article.

L'adsorption chromatographique a beaucoup facilité l'étude des pigments au cours des dernières années; pour des détails sur cette méthode voir Lederer (1939 *a*), Willstädt (1939) et Zechmeister & Cholnoky (1937).

## II. CAROTÉNOÏDES

Nous ne traiterons ici que des résultats importants acquis depuis 1938. De nombreux articles de revue et des monographies décrivent la chimie et la biochimie de ces corps (Karrer, 1932; Lederer, 1934, 1935, 1938 *a, b*; Zechmeister, 1934, 1937).

L'instabilité des caroténoïdes, mise récemment en évidence, rend leur étude extrêmement délicate. Des caroténoïdes parfaitement homogènes gardés pendant quelque temps en solution, s'isomérisent spontanément (Zechmeister & Tuszon, 1938; Gillam & El Ridi, 1936; Gillam *et al.* 1937). Cette isomérisation est plus rapide à température élevée ou en présence de traces d'iode (Zechmeister & Tuszon, 1939). De plus l'action d'acides dilués transforme le carotène en un mélange de quatre ou cinq pigments nouveaux (Quackenbush *et al.* 1938). Enfin Kuhn & Sörensen (1938) ont décrit un caroténoïde qui s'oxyde rapidement à l'air en solution alcaline (voir ci-dessous). Ces travaux prouvent la grande réactivité de la chaîne polyénique des lipochromes.

L'irradiation d'une suspension de gamètes de *Chlamydomonas eugametos* provoque d'abord la formation de cils, puis la copulation. Une goutte d'une suspension irradiée déclenche le même phénomène dans une suspension gardée dans l'obscurité; il y a donc formation d'un ou de plusieurs corps dont la présence est indispensable à la reproduction de ces organismes. Kuhn *et al.* (1938) ont constaté les faits suivants: les cils des gamètes, immobiles d'abord, deviennent mobiles en présence d'un pigment hydrosoluble qui n'est rien d'autre que la *crocine* (digentiobioside de la crocétine, I). En continuant l'irradiation, ce sont d'abord les gamètes femelles qui

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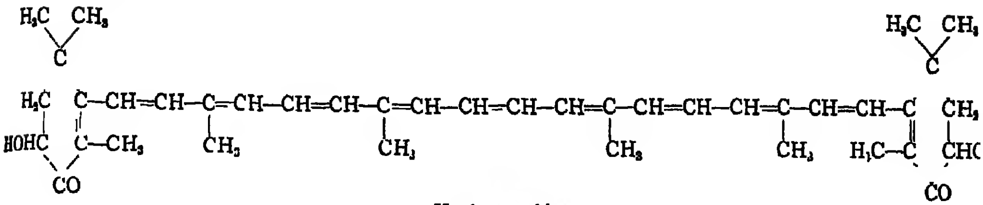
I. Crocétine

deviennent aptes à la copulation, puis les gamètes mâles. Ce phénomène doit être attribué à deux "facteurs de copulation" formés par irradiation de la crocine; ce ne sont pas des corps homogènes, mais des mélanges de deux corps dont l'un se forme d'abord, l'autre ensuite. Le premier est identique à un éther (diméthylique?) de la *cis-crocétine*, le deuxième à un éther de la *trans-crocétine*. Un mélange de trois parties d'éther *cis* avec une partie d'éther *trans* rend les gamètes femelles aptes à la copulation, tandis que le mélange inverse provoque les mêmes effets chez les gamètes mâles. Nous observons ici une nouvelle action biologique des caroténoïdes et en même temps de curieuses relations entre action biologique et configuration stérique (voir aussi Moewus, 1939).

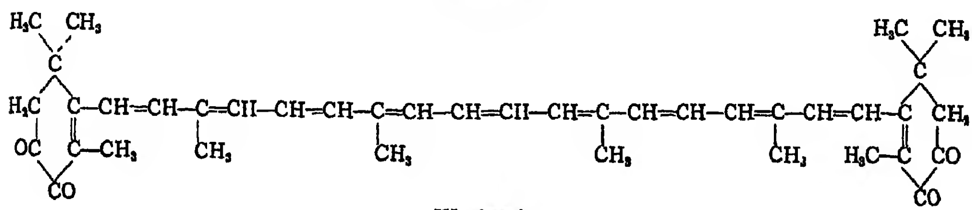
Cette action des caroténoïdes sur la reproduction ne s'observe peut-être chez aucun Invertébré, mais le contraire est plus probable; nombreux sont, en effet, les œufs ou les glandes sexuelles des Invertébrés contenant des caroténoïdes (par exemple: *Pecten maximus*, *Pectunculus glycymeris*, *Paracentrotus lividus* (Lederer, 1938 *b*)—pour *Astacus gammarus*, voir ci-dessous).

*Astaxanthine et ovoverdine.* Kuhn & Sörensen (1938) ont trouvé que "l'ester hypophasique" de l'astacine ("ovoester") isolé par Kuhn & Lederer (1933), à partir

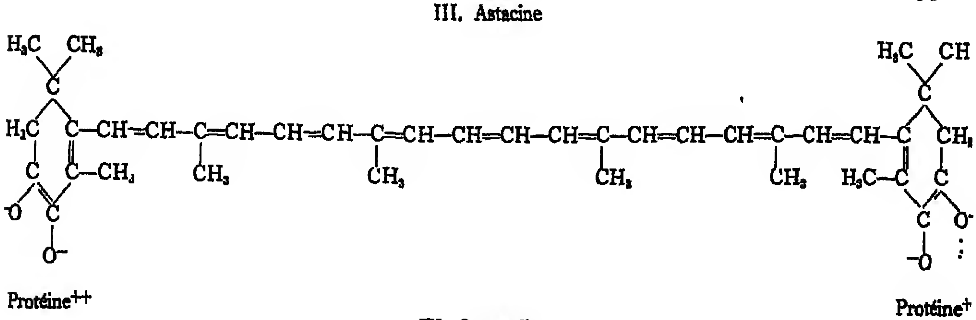
des œufs de Homard, n'est pas un ester, mais une xanthophylle $C_{40}H_{52}O_4$ avec deux groupes —OH et deux groupes —CO (II), qui se transforme par oxydation en milieu alcalin en astacine $C_{40}H_{48}O_4$ (III). Pour la constitution de l'astacine voir Karrer & Benz (1934), Karrer & Loewe (1934) et Karrer *et al.* (1935). Cette xanthophylle s'appelle dorénavant "astaxanthine". L'ester épiphasique de l'astacine de la carapace et de l'hypoderme du Homard est en vérité un ester de l'astaxanthine qui s'oxyde au cours de la saponification pour donner de l'astacine (III). L'astacine ne serait donc pas un pigment naturel, mais résulterait d'une déshydrogénation de l'astaxanthine.



II. Astaxanthine



III. Astacine



IV. Ovoverdine

A l'abri de l'air, l'astaxanthine donne un sel de potassium bleu foncé ayant une constitution diénolique. Sa couleur rappelle celle de l'*ovoverdine*, pigment bleu-vert, hydrosoluble, des œufs du Homard. L'ovoverdine aurait, selon Kuhn & Sörensen, la constitution (IV). La protéine est liée avec le diénol de l'astaxanthine sous forme d'un sel et en outre par des valences partielles.

Peu avant, Stern & Salomon (1938) avaient étudié les propriétés de ce chromoprotéide et souligné les analogies qu'il présente avec le pourpre visuel rétinien des Vertébrés. Wyckoff (1937) a mesuré sa constante de sédimentation qui indique un poids moléculaire de 300,000. D'après Stern & Salomon, une molécule d'ovoverdine contient une molécule de caroténoïde. D'après Kuhn & Sörensen, le poids moléculaire de l'ovoverdine ne serait que 144,000 (déterminé par voie chimique).

Stern & Salomon ont mis en évidence une dissociation thermique réversible de l'ovoverdine. Une solution verte chauffée à 70° vire au rouge; après refroidissement la couleur verte de ce chromoprotéide réapparaît.

Il existe chez des Anémones de mer des pigments ressemblant à l'astaxanthine, mais dont la bande d'absorption, mesurée après saponification, c'est-à-dire après oxydation est décalée un peu vers le bleu par rapport à celle de l'astacine. Heilbron *et al.* (1935) en ont trouvé dans *Actinoloba dianthus* (495 mμ. dans $CS_2$, p. f. 196°) et dans *Tealia felina* (500 mμ. dans $CS_2$, p. f. 208°), et Fox & Moe (1938) en ont décrit dans *Epiactis prolifera* avec une bande également à 500 mμ. (dans $CS_2$). La bande de l'astacine est à 510 mμ. dans $CS_2$.

La découverte de la vraie nature de l'astaxanthine rend encore plus probable sa formation à partir de la taraxanthine, $C_{40}H_{56}O_4$ discutée par plusieurs auteurs (Burkhardt *et al.* 1934; Fox & Moe, 1938; Lederer, 1938*a*).

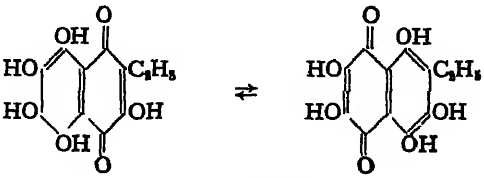
## III. QUINONES

### (1) *Naphtoquinones (pigments d'Oursins)*

MacMunn (1885*a*) a observé que les élaeocytes du liquide périvisceral des Oursins, *Strongylocentrotus lividus*, *Amphidotus cordatus*, *Echinus esculentus* et *E. sphaera*, contiennent un pigment rouge qu'il appela *échinochrome*. Ce corps étant décoloré par des agents réducteurs, il lui attribua des fonctions de pigment respiratoire. Griffiths (1892*a*) prétend avoir obtenu de l'haematoporphyrine et de l'hémochromogène par hydrolyse du pigment. Plus tard, McClendon (1912) a trouvé le même pigment dans les élaeocytes, les œufs et le test de l'Oursin, *Arbacia punctulata*. Le premier, il en obtint des cristaux. Il n'a pas pu confirmer le rôle respiratoire ni la présence de fer et a reconnu la nature acide du pigment. Cannan (1927) a trouvé que l'hydrosulfite décolore l'échinochrome et a mesuré son potential d'oxydo-réduction (potentiel normal $+0{\cdot}1995$ volt; $-0{\cdot}230$ volt à *p*H 7 d'après Friedheim, 1933). Ni lui, ni Cook (1928) n'ont confirmé l'absorption d'oxygène par l'échinochrome. Ce pigment se trouve cependant dans le sang d'*Echinus* à l'état réduit, tandis qu'il est à l'état oxydé dans les œufs et le test d'*Arbacia*. Vlès & Vellinger (1928) ont étudié les changements de couleur et de spectre d'absorption à différents *p*H de l'échinochrome ("arbacine") extrait des œufs d'*Arbacia aequituberculata*. Leur pigment était orangé à *p*H 2·4–3·0, violacé de 4 à 7 et jaune à partir de *p*H 8. Ils préconisent l'emploi de ce pigment comme indicateur de *p*H intracellulaire et concluent que le *p*H de l'œuf d'*Arbacia* est de l'ordre de $5{\cdot}5 \pm 0{\cdot}3$.

Ball (1936), puis Lederer & Glaser (1938), ont isolé l'échinochrome des œufs d'*Arbacia aequituberculata* à l'état cristallisé et ont établi sa formule brute, $C_{12}H_{10}O_7$. Le même pigment se trouve dans les épines et le test de *Paracentrotus lividus* accompagné d'un autre pigment, plus foncé, appelé *spinochrome*. En 1939, Glaser & Lederer ont décrit plusieurs dérivés méthylés de l'échinochrome et du spinochrome et ont étudié la distribution de ces deux corps et de quelques autres pigments analogues qui les accompagnent. L'intérêt de ce groupe de pigments

naturels s'est considérablement accru par la découverte de Hartmann *et al.* (1939) que l'échinochrome secrété par les œufs murs d'*Arbacia pustulosa* active les mouvements des spermatozoaires et les attire vers l'œuf. Kuhn & Wallenfels (1939) ont établi sa constitution chimique. C'est une 2 éthyle, 1:4 naphtoquinone portant cinq groupes hydroxyles occupant toutes les places libres du noyau naphtalénique (V).



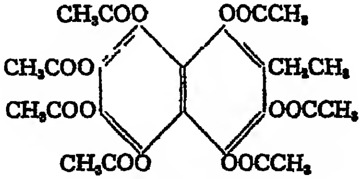
V. Échinochrome

Kuhn & Wallenfels proposent d'appeler les pigments des œufs d'Oursins "échinochromes" et les pigments des épines "spinochromes" en ajoutant des lettres majuscules comme suffixe pour indiquer l'espèce dont le pigment a été préparé. Ils appellent le pigment des œufs d'*Arbacia* "échinochrome A" et celui des épines de *Paracentrotus* "spinochrome P". Nous ne voyons pas la nécessité d'appliquer cette nomenclature compliquée qui est d'autant moins justifiée que l'échinochrome et le spinochrome sont des pigments très bien définis et faciles à caractériser. Nous continuerons donc, d'accord avec Monsieur P. Karrer, d'employer les noms échinochrome et spinochrome sans suffixe.

*Échinochrome.* Après extraction par l'acétone ou de l'alcool acidulé on fait passer le pigment dans l'éther éthylique, d'où on l'extrait avec une solution de bicarbonate, grâce à ses fonctions phénoliques; après acidification, on le reprend par l'éther et le purifie ensuite par adsorption chromatographique sur le carbonate de calcium et par sublimation dans le vide poussé (Lederer & Glaser, 1938).

L'échinochrome cristallise sous forme d'aiguilles rouge foncé groupées souvent en rosettes et fondant à 220°; son spectre d'absorption présente trois bandes situées à 526, 490, 456 m$\mu$. ($CHCl_3$). Les cristaux sont facilement solubles dans l'alcool, l'éther, l'acétone; insolubles dans l'éther de pétrole et dans l'eau. L'échinochrome a quatre atomes d'hydrogène mobiles dont deux sont acides. Il donne avec le trichlorure de fer une forte coloration vert-brune.

La constitution chimique ressort des expériences de Kuhn & Wallenfels (1939). La distillation sur la poudre de zinc donne du naphtalène et l'acétylation réductive, un dérivé heptacétylé du leuco-échinochrome (VI). Le groupe éthyle forme de

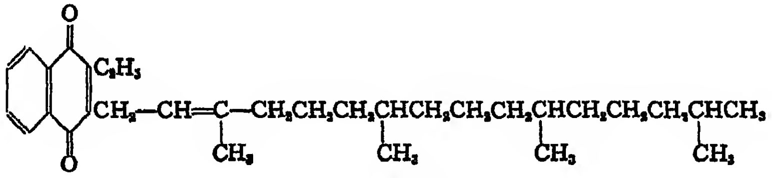


VI. Hepta-acétyl-leuco-échinochrome

l'acide propionique par oxydation chromique. L'échinochrome lui-même serait représenté par les deux 1:4 naphtoquinones en équilibre (V).

Lillie (1919) a montré dans une série de mémoires que l'œuf mûr excrète des substances qui provoquent l'activation et l'agglutination des spermatozoïdes. (Pour les détails de sa théorie de "fertilisine", voir Just, 1930.) Lillie savait déjà que le corps provoquant l'agglutination n'est pas identique à celui qui provoque l'activation et l'attraction. Ceci a été confirmé par Hartmann *et al.* (1939) qui identifient le dernier corps avec l'échinochrome. Sa présence serait décelable à une concentration de 1 à 20 milliards. D'après Kuhn & Wallenfels (1939) l'échinochrome catalyserait certaines réactions chimiques chez le spermatozoaire. Tyler (1939) n'a pu constater aucun effet de l'échinochrome sur la respiration des œufs et des spermes de *Strongylocentrotus*.

Les pigments du groupe de l'échinochrome sont les premiers dérivés de la naphtoquinone trouvés chez des animaux. Des recherches d'un autre domaine apportent en même temps une lumière nouvelle sur l'importance des naphtoquinones dans la vie des animaux. Nous pensons aux nombreux travaux récents sur la vitamine K (antihémorragique). La vitamine K, elle-même, serait d'après Corquodale *et al.* (1939) une 2 éthyle, 3 phytyle, 1:4 naphtoquinone (VII). Voir aussi Karrer & Geiger (1939). L'échinochrome n'agit pas comme vitamine K (Kuhn *et al.* 1939).



VII. Vitamine $K_1$

*Spinochrome*. Les épines violettes de *Paracentrotus lividus* contiennent un pigment rouge foncé que nous avions obtenu à l'état cristallisé il y a quatre ans (voir Lederer, 1938*b*). Moore (1937) mentionne un travail non publié de Stern qui a isolé des cristaux de ce pigment qu'il appelle échinorubine. Lederer & Glaser (1938) ont séparé ce pigment par adsorption chromatographique, en échinochrome et en spinochrome, pigment plus foncé et moins facile à cristalliser; on le purifie d'après les méthodes éprouvées pour l'échinochrome.

Le spinochrome cristallise à partir d'éther acétique sous forme de priames violet-foncé, presque noirs qui fondent vers 185° et qui présentent trois bandes d'absorption situées à 581, 538 et 496 m$\mu$. (dans $CHCl_3$). Ils sont moins solubles que ceux de l'échinochrome. Le spinochrome est adsorbé sur la carbonate de calcium en une zone bleu-violette au-dessus de la zone brune d'échinochrome (voir aussi Tyler, 1939).

La formule brute du spinochrome est $C_{12}H_{10}O_8$. Il a cinq atomes d'hydrogène mobiles, est décoloré par l'hydrosulfite, comme l'échinochrome, et donne une coloration foncée avec le trichlorure de fer. Sa composition élémentaire ainsi que ses propriétés font penser qu'il dérive de l'échinochrome par substitution d'un groupe hydroxyle à un atome d'hydrogène; il se peut pourtant que le spinochrome soit plus insaturé que l'échinochrome.

Glaser & Lederer (1939) ont préparé trois dérivés méthylés du spinochrome qui cristallisent facilement.

*Pigments associés.* Deux autres pigments de ce groupe ont été obtenus à l'état cristallisés à partir des épines d'*Arbacia*. Ce sont l'*isoéchinochrome* de Glaser & Lederer (1939) (fines aiguilles brun-rouge, p. f. 247°, mieux adsorbé que l'échinochrome; bandes d'absorption à 548 et 510 m$\mu$. dans $CHCl_3$) et un pigment analogue isolé par Kuhn & Wallenfels (1939) (feuillets rouge-foncé, p. f. 229°; 555, 515, 465 m$\mu$. dans $CS_2$). Il se peut que les deux corps soient identiques.

Glaser & Lederer (1939) ont trouvé dans *Arbacia* et *Paracentrotus* quelques autres pigments, rouges et bruns, peu solubles dans l'éther, mais solubles dans l'alcool butylique. Leur appartenance au groupe de l'échinochrome se manifeste par leur solubilité dans le bicarbonate avec une couleur foncée et par leur réaction avec le trichlorure de fer.

Le test des *Paracentrotus* verts contient un pigment jaune présentant une vive fluorescence bleue qui vire au vert en solution bicarbonatée.

Les pigments dérivés de la naphtoquinone d'origine végétale diffèrent des pigments d'Oursin en ce qu'ils ont beaucoup moins de groupes hydroxyles. Il faut croire que les chinones sont synthétisés par les Oursins. On n'en a pas encore trouvé chez d'autres espèces d'animaux, pas même chez d'autres Échinodermes.

Les colorations diverses des épines et tests de *Paracentrotus lividus* sont dues à des mélanges de spinochrome et d'échinochrome en différentes proportions. Voici un tableau d'après Glaser & Lederer (1939).

| Couleurs des épines | Verdâtre | Vert brun et brun violet | Violet |
|---|---|---|---|
| Mg. d'échinochrome par animal (test et épines) | 0·5 | 0·2 | 0·02 |
| Mg. de spinochrome par animal (test et épines) | 0·06 | 0·8 | 1·7 |

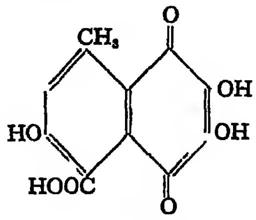
Ces pigments ne se trouvent pas seulement dans la partie calcaire des épines et du test, mais également dans le derme avec ses pieds ambulacraux et pédicillaires, remplis de granulations colorées. Le tableau suivant montre la distribution des différents pigments dans les organes de cinq espèces d'Oursins (d'après des essais de Glaser & Lederer, en partie inédits).

| Espèce | Ovaires | Derme | Test et épines |
|---|---|---|---|
| *Arbacia aequituberculata* | Échinochrome | — | Isoéchinochrome |
| *Paracentrotus lividus* (variété verte) | Caroténoïdes | Échinochrome | Échinochrome, et un peu de spinochrome |
| *Paracentrotus lividus* (variété violette) | Caroténoïdes | Échinochrome Spinochrome | Spinochrome, et un peu d'échinochrome |
| *Echinus esculentus* | Peu colorés | Échinochrome | Échinochrome |

### (2) *Anthraquinones (cochenille, kermès et acide laccaïnique)*

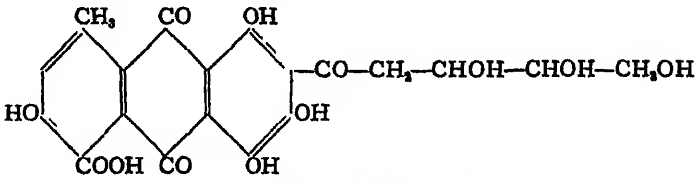
Les colorants de ce groupe sont produits par des Insectes. Nous devons des connaissances précises sur leur structure à Dimroth et ses collaborateurs. On ne sait rien de leur rôle biologique.

*L'acide carminique*, $C_{22}H_{20}O_{13}$, est le colorant de la "cochenille"; on l'extrait des femelles de *Coccus cacti*, vivant sur des cactées de l'Amérique centrale. L'acide carminique forme des cristaux rouge-foncé, se décomposant vers 205°. La distillation sur la poudre de zinc donne de l'anthracène et de l'α-méthylanthracène. L'oxydation par le permanganate fournit la carminazarine (VIII) (Dimroth &



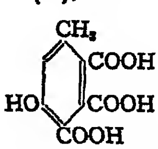

VIII. Carminazarine

Kerkovius, 1913), ce qui fixe la constitution des deux premiers noyaux. Le troisième noyau porte trois groupes hydroxyles et une chaîne latérale, —CO—$CH_2$—CHOH—CHOH—CHOH—$CH_2$OH, qui est la cause de l'activité optique de l'acide carminique (IX) (Dimroth, 1909; Dimroth & Kämmerer, 1920).



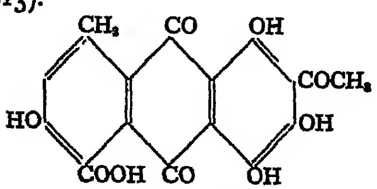

IX. Acide carminique

*L'acide kermésique*, $C_{18}H_{12}O_9$, est le pigment du kermès, colorant composé des corps séchés de *Coccus ilici* vivant sur des chênes dans les pays méditerranéens. La distillation du pigment sur le zinc donne le α-méthylanthracène, l'oxydation par le permanganate l'acide cochenillique (X), comme l'acide carminique. Les deux



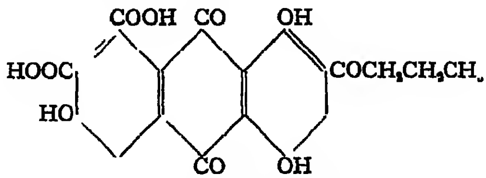

X. Acide cochenillique

pigments ne diffèrent que par la chaîne latérale qui est constituée dans l'acide kermésique par le groupe acétyle (XI) (Dimroth, 1910; Dimroth & Fick, 1916; Dimroth & Scheurer, 1913).




XI. Acide kermésique

*L'acide laccaïnique*, $C_{20}H_{14}O_{10}$, se trouve dans la laque sécrétée par l'arbre à laque après piqûre par *Coccus laccae*. Le pigment est excrété par des Insectes au moment de la piqûre. La formule brute de ce pigment est $C_{20}H_{14}O_{10}$ (Dimroth & Goldschmidt, 1913). Il s'agit également d'un dérivé de l'anthraquinone pour lequel Bergmann (1933) propose la formule XII.



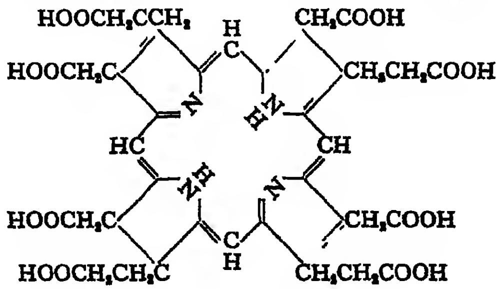
XII. Acide laccaïnique

## IV. PIGMENTS PYRROLIQUES

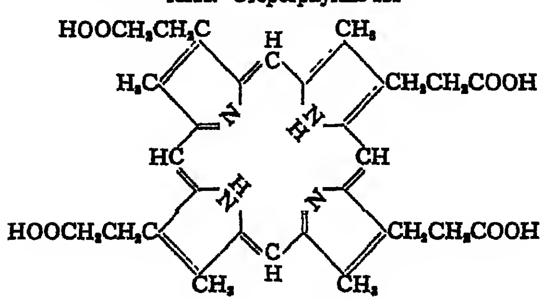
### (1) *Porphyrines*

Les porphyrines sont des pigments rouges ou bruns dont le rôle essentiel dans la nature est de lier le fer sous forme de sel complexe et de former ainsi la base des pigments respiratoires. La description de ces derniers sort du cadre de la présente revue (voir Florkin, 1934*a*, *b*; Redfield, 1933; Roche & Combette, 1937. Pour la chimie des porphyrines, voir Fischer & Orth, 1937).

*Conchoporphyrine* ($C_{42}H_{48}O_{10}N_4$). Fischer & Jordan (1930) ont isolé ce pigment à partir des coquilles du Lamellibranche *Pteria radiata*. Son spectre d'absorption ressemble à celui de l'uroporphyrine, forme d'excrétion des porphyrines dans l'organisme animal, contenant quatre groupes d'acide acétique et quatre groupes d'acide propionique (XIII). La conchoporphyrine contient trois groupes carboxyles de moins et se transforme avec perte d'un groupe carboxyle en coproporphyrine



XIII. Uroporphyrine III



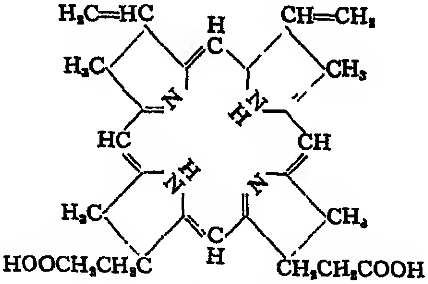
XIV. Coproporphyrine III

(XIV) également une des porphyrines des excrétions des animaux à hémoglobine. L'instabilité d'un groupe carboxyle avait fait penser à Fischer qu'il s'agissait d'un groupement $—CH_2CH{=}(COOH)_2$ (dérivé de l'acide malonique), mais une porphyrine contenant ce groupement, synthétisée par Fischer & Holt (1934) n'est pas identique à la conchoporphyrine. Il faut croire qu'il s'agit plutôt d'un groupe succinique (voir aussi Fischer & Hofmann, 1937).

L'éther pentaméthylique de la conchoporphyrine cristallise sous forme de prismes brun-rouge fondant à 271–273°. Leur spectre d'absorption montre cinq bandes (626·5, 599·8, 571·4, 536·3, 506·5 mμ. dans $CHCl_3$). La conchoporphyrine a une forte fluorescence rouge (spectre de fluorescence: 600, 626, 659, 676, 695·5 mμ. dans le dioxane). Fischer & Haarer (1931) ont isolé l'uroporphyrine I à partir des coquilles de *Pteria vulgaris*. D'après Waldenström (1937) il s'agit d'un mélange des uroporphyrines I et III. L'autres porphyrines s'y trouvent en petite quantité.

*Porphyrines tégumentaires*. MacMunn (1886) a signalé la présence de porphyrines dans les téguments d'*Uraster rubens*, d'*Arion empiricorum*, dans les Étoiles de mer brunes, et chez divers Cœlentérés (*Flabellum variabile*, *Fungia symmetrica*). Le caractère porphyrinique à été établi par l'étude du spectre d'absorption, de la fluorescence rouge et de la solubilité. Dhéré & Baumeler (1928*b*) ont confirmé la présence d'une porphyrine à fluorescence rouge dans le derme d'*Arion empiricorum*.

La porphyrine de la peau du Ver de terre est la protoporphyrine (XV) très



XV. Protoporphyrine III

répandue chez les Vertébrés (dans les fèces, dans la moelle épinière des malades d'anémie pernicieuse, dans la viande pourrie, dans les coquilles de beaucoup d'œufs d'oiseaux—ooporphyrine—) et dans les feuilles de beaucoup de plantes (Fischer & Hilmer, 1925). On peut donc penser que la porphyrine tégumentaire de *Lumbricus terrestris* provient de sa nourriture végétale. Il se peut que les porphyrines déposées dans l'épiderme des animaux jouent un rôle de photo-sensibilisateurs (Hausmann, 1916).

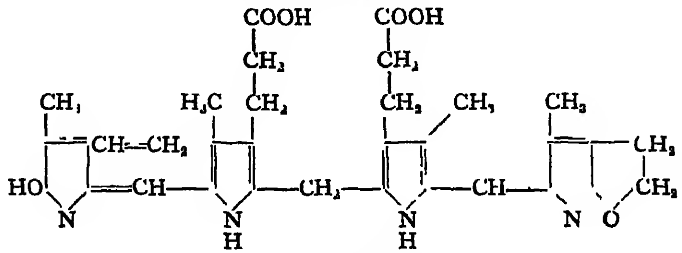
Un pigment analogue à celui du Ver de terre se trouve dans le tégument d'*Eisenia foetida* (Hausmann, 1916).

Chez les Helminthes parasites, Derrien (1927) a trouvé la protoporphyrine dans le tégument des Cysticerques de *Taenia solium*, et Aduco (1888) semble avoir constaté la présence de porphyrines dans un *Eustrongylus gigas* de chien, coloré en rouge. Dans ces cas c'est évidemment l'hémoglobine du vertébré qui est l'origine de la porphyrine.

Fischer & Fink (1925) ont trouvé la protoporphyrine et la coproporphyrine dans les fèces des chenilles de mite.

(2) *Pigments biliaires*

Les pigments biliaires sont, chez les Vertébrés, le résultat de la destruction du pigment sanguin. Ce processus commence par la rupture du noyau porphyrinique à un endroit déterminé et finit par l'élimination du fer (Lemberg, 1935). Les pigments biliaires, dont le plus important est la bilirubine (XVI), diffèrent des

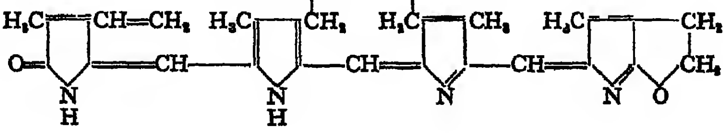


XVI. Bilirubine

porphyrines par l'absence de bandes d'absorption caractéristiques et de la fluorescence rouge. Ils ont conservé la faculté de former des sels complexes avec les métaux; leurs sels de zinc présentent le plus souvent des fluorescences rouges ou vertes ("réaction urobilino-zincique"). (Voir Javillier, 1926; Fischer & Orth, 1937; Dhéré, 1938.) Normalement, chez les Vertébrés les pigments biliaires se forment dans le foie, mais leur formation extrahépatique est également fréquente. Chez les Invertébrés, les organes hépatiques, et leurs sécrétions semblent souvent dépourvus de pigments biliaires (Roaf, 1906). La bile de quelques Mollusques et Crustacés contient de l'hélicorubine, pigment hématinique qui ne semble pas se transformer en pigments biliaires (Vegezzi, 1916; Anson & Mirsky, 1925).

Pour les Invertébrés à sang hémoglobinique, notons l'observation de Spiess (1910) sur une bilirubine dans les "cellules hépatiques" de la Sangsue (*Hirudo medicinalis*), de Gheorghiu (1933) sur un pigment biliaire chez l'Hirudinée, *Protoclepsis tesselata*. Bloch-Raphael (1939) a trouvé par des méthodes histochimiques que de nombreux Vers polychètes contiennent des pigments biliaires, qui paraissent non seulement être les produits de destruction de l'hémoglobine, mais aussi, dans quelques cas, des précurseurs de celle-ci.

La "pontobdelline", pigment tégumentaire d'un bleu verdâtre de l'Hirudinée *Pontobdella*, présente d'après Abeloos (1925) les caractères de solubilité de la biliverdine (XVII) et ses solutions donnent la réaction de Gmelin, caractéristique des pigments biliaires. Les Pontobdelles se nourissant de sang de Vertébrés, leur pigment peut être dérivé de l'hémoglobine.



Abeloos (1925) rapporte encore la présence de pigments tégumentaires rouges ou bruns chez les Hirudinées *Glossiphonia complanata*, *G. heteroclita*, *Hemiclepsis marginata*, et *Piscicola geometra*.

MacMunn (1885 *b*) a trouvé une biliverdine dans le mésoderme d'*Actinia mesembryanthemum* à côté d'une hématine.

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D'après von Linden (1903) les écailles des ailes des Vanesses (Lépidoptères) contiennent un chromoprotéide rouge dont le groupe prosthétique donne la réaction de Gmelin; le pigment serait formé dans l'intestin de la chenille à partir de la chlorophylle. Porta (1902) a signalé l'excrétion d'un pigment voisin de l'urobiline chez les Coccinelles.

Nous traiterons maintenant de quelques pigments spécifiques dérivés probablement des pigments biliaires.

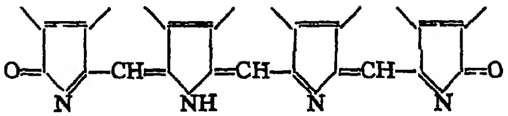
*Rufine.* Dhéré & Baumeler (1928 *a*) ont donné le nom de rufine au pigment rouge du tégument de la limace rouge (*Arion rufus*). Il ne s'agit pas d'un caroténoïde comme on l'avait cru (Bohn, 1901; Mandoul, 1903). Dor (1902) le désigne sous le nom d'urobiline; Schulz (1904) conclut qu'il n'y a pas d'urobiline, ni de pigment semblable.

Dhéré & Baumeler (1928 *a*) ont trouvé que le pigment, jaune à l'état neutre ou faiblement acide, vire au rouge par addition d'acides forts. En chauffant à la lumière une solution fortement acidulée, elle vire au vert. Alcalinisée par la soude, la rufine vire au rouge violacé. Les solutions de rufine, acides ou alcalines, sont décolorées par réduction à l'hydrosulfite; la couleur réapparaît après aération.

Dhéré *et al.* (1928), en étudiant la spectrochimie de la rufine, ont trouvé une forte bande à 430 m$\mu$. pour une solution faiblement acide, une absorption terminale croissante jusqu'à 360 m$\mu$. pour une solution alcaline rouge violacée et deux bandes (502 et 468 m$\mu$.) pour une solution fortement acide. La solution verte obtenue par traitement à l'acide sulfurique concentré présente trois bandes (650, 580, 471 m$\mu$.). Avec de l'acide plus fort la liqueur prend une forte coloration rougeâtre et montre deux bandes d'absorption (542, 503 m$\mu$.).

*Rufescine.* Krukenberg (1883, 1886) a décrit sous le nom de turbobromine un pigment rouge de la coquille de certains Gastéropodes (*Haliotis rufescens*, *Turbo olivaceus*, *T. sarmaticus*, *T. radiatus*, *T. petholatus*, *T. maximus*). Ce pigment, soluble dans l'alcool acidulé est accompagné d'un pigment vert qui donne la réaction de Gmelin et semble identique au pigment vert obtenu par oxydation du pigment rouge. Schulz (1904) a consacré un long mémoire aux pigments biliaires des coquilles de Mollusques et a confirmé en général les données de Krukenberg. Plus tard, Dhéré & Baumeler (1930) se sont occupés en détail du pigment de la coquille de l'*Haliotis rufescens* qu'ils appellent *rufescine*.

Les solutions aqueuses de rufescine, faiblement acides sont relativement stables (bandes d'absorption à 518, 450 m$\mu$. environ). L'addition d'acide acétique fait virer au jaune, puis au rouge ("haliotirubine", bandes d'absorption à 525, 455 m$\mu$. environ). L'action d'acides forts produit un pigment vert ("haliotiverdine", pas de bandes d'absorption). Cette même coloration apparaît par l'action de trichlorure de fer ou d'eau oxygénée en milieu faiblement acide sur la rufescine. Une solution d'haliotiverdine, alcalinisée, vire au jaune; en acidifiant et en agitant constamment Dhéré & Baumeler ont obtenu un nouveau dérivé de couleur bleue ("halioticyanine", bandes: 620, 572 m$\mu$.). Son spectre est analogue à celui de la "bilicyanine" (phase bleue de la réaction de Gmelin) (XVIII). Les solutions de rufescine, d'halio-



XVIII. Phase bleue de la réaction de Gmelin

tirubine et d'haliotiverdine ne possèdent aucune fluorescence, mais l'haliotirubine en présente une de couleur rouge très nette (comme la bilicyanine). De plus, Dhéré & Baumeler ont obtenu comme dernier stade d'oxydation de la rufescine un dérivé dont les caractères spectraux, ainsi que ceux du sel de zinc, correspondent de très près à ceux de l'urobiline. La rufescine elle-même forme un sel complexe de zinc avec une fluorescence verte et une forte bande d'absorption dans le bleu, comme l'urobiline.

*Pigments d'*Haliotis californiensis. Les coquilles du Gastéropode *Haliotis californiensis* sont colorées en bleu-vert. D'après Schulz & Becker (1931) il s'agirait tout simplement d'indigo.

Lemberg (1931) conteste ceci et montre qu'il y a deux pigments, un corps *A* qui est vert dans le chloroforme acide (bandes à 623, 498 m$\mu$.) et un autre, *B*, pigment principal, qui est bleu (622 m$\mu$.). Le pigment *B* a une fluorescence rouge, et *A* et *B* forment des sels de zinc. Ce sont des corps acides qui se trouvent dans la coquille sous forme de sels de calcium. Lemberg pense que ces pigments ont bien une structure pyrrolique.

*Calliactine*. L'Anémone de mer, *Sagartia parasitica* (*Calliactis effoeta*), possède plusieurs pigments décrits par Abeloos & Teissier (1926). Le plus intéressant est le pigment de la colonne qui se présente sous forme de groupes de grains rouges dans la partie superficielle de la paroi et de groupes de grains violets dans les couches plus profondes. Ce pigment est un véritable indicateur de *p*H. Les acides le font passer du jaune-brun au jaune d'or, puis au rouge. Les alcalis le font passer au bleu, puis au violet et finalement au rouge. Ce pigment est soluble dans l'eau et les alcools, insoluble dans l'éther et le chloroforme. On l'obtient à l'état cristallisé en ajoutant de l'alcali à une solution alcoolique (rosettes d'aiguilles bleues, ne fondant pas jusqu'à 300°) (Lederer *et al.* 1940). Ce pigment, la "calliactine", $C_{22}H_{22}O_6N_4$ forme un chlorhydrate rouge; chauffé avec de l'acide chlorhydrique à 10 % il se

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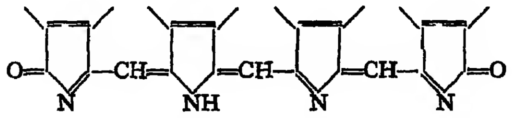

XVIII. Phase bleue de la réaction de Gmelin

tirubine et d'haliotiverdine ne possèdent aucune fluorescence, mais l'haliotirubine en présente une de couleur rouge très nette (comme la bilicyanine). De plus, Dhéré & Baumeler ont obtenu comme dernier stade d'oxydation de la rufescine un dérivé dont les caractères spectraux, ainsi que ceux du sel de zinc, correspondent de très près à ceux de l'urobiline. La rufescine elle-même forme un sel complexe de zinc avec une fluorescence verte et une forte bande d'absorption dans le bleu, comme l'urobiline.

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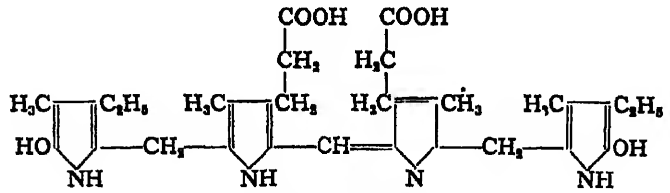
transforme en "chloro-calliactine" ($C_{22}H_{18}O_2N_4Cl_2$; aiguilles vert foncés). Ce nouveau pigment perd son chlore par chauffage avec de l'eau et se transforme en "néo-calliactine" ($C_{22}H_{18}O_3N_4$; aiguilles vert-jaunes, ne fondant pas jusqu'à 300°).

Chloro-calliactine et néo-calliactine sont violettes en milieu alcalin et ont, comme la calliactine, une fluorescence orange. La réaction de Gmelin est négative, mais la composition chimique ainsi que quelques réactions de ces corps font penser qu'il s'agit bien de dérivés de pigments biliaires.

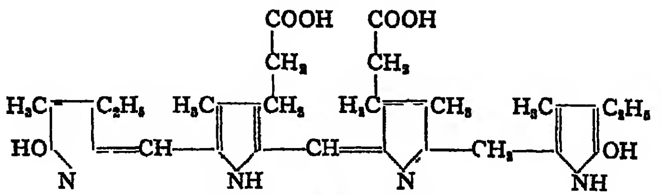
*Pigments de la sécrétion des Aplysies.* Les Aplysies (Limaces de mer, *Aplysia punctata*, *A. depilans*, *A. limacina*) secrètent par des glandes spéciales un pigment d'un violet intense, accompagné de mucus et d'une matière malodorante. Le pigment, l'aplysiopurpurine, a depuis longtemps suscité l'intérêt des chercheurs.

D'après Ziegler (1886) il ne s'agit de rien d'autre que du rouge d'aniline et du violet d'aniline. Les frères Negri (1875) montrent l'erreur de Ziegler et constatent que le colorant violet vire au bleu par addition d'acide et devient soluble dans le chloroforme. De nombreuses observations de Moseley (1877) et de MacMunn (1899) font ressortir la nature complexe de ce pigment. Paladino (1908) affirme qu'il contient du fer et une trace de manganèse. Derrien & Turchini (1925) signalent la fluorescence rouge du liquide sécrété et la fluorescence verte de la solution alcoolique oxydée; ceci les incite à rapprocher l'aplysiopurpurine des porphyrines. Schreiber (1932) sépare ce pigment en une "urobiline" (XIX) et un produit qui donne "de



XIX. Urobiline

l'urobiline" par oxydation. Enfin, Fontaine & Raffy (1936) dans une étude de la spectrochimie de l'aplysiopurpurine concluent que ce pigment se rapproche de très près de la mésobilivioline (XX). Ils pensent, comme Schreiber (1932), qu'elle



XX. Mésobilivioline

pourrait être un produit de dégradation non seulement de la chlorophylle, mais aussi des phycochromoprotéides.

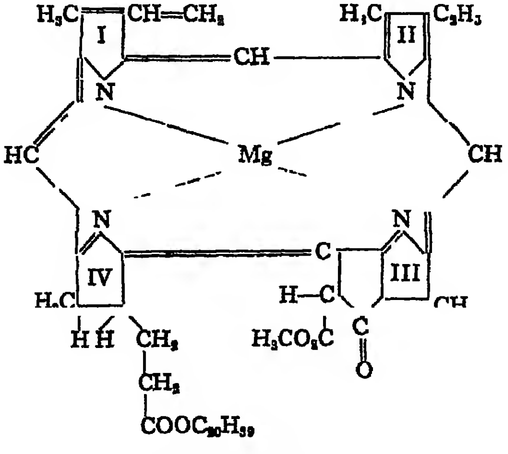
Nous avons trouvé que l'aplysiopurpurine se comporte comme un mélange de

chromoprotéides que l'on peut séparer par précipitation fractionnée par le sulfate d'ammonium ou par adsorption chromatographique sur l'alumine (Lederer & Huttrer, 1939). Bien que Ziegler (1886) et MacMunn (1889) aient déjà trouvé que le pigment peut être précipité par des sels neutres, aucun des auteurs cités n'a tiré les conclusions appropriées de cette observation. Les chromoprotéides en question sont assez instables; ils dissocient par action de la température et de la lumière. La dissociation thermique est réversible comme pour l'ovoverdine. Les groupes prosthétiques des protéides en question sont solubles dans l'alcool butylique. On peut les séparer par adsorption sur la chaux ou le talc en sept ou huit constituants différents qui rappellent d'après leurs propriétés chimiques et physiques (composition élémentaire, réaction de Gmelin, spectres en solution acide, neutre ou à l'état de sel complexe de Zn) la mésobilivioline, la mésobilirhodine (isomère de la première), l'urobiline et autres pigments biliaires analogues.

*Pigment vert des ailes de* Pieris brassicae. Les ailes du Piéride du choux contiennent un pigment vert, hydrosoluble (Hopkins, 1892). On peut le séparer en un corps vert et un corps bleu par fractionnement au sulfate d'ammonium (Wieland & Kotzschmar, 1937). Il s'agit de chromoprotéides dont le groupe prosthétique, soluble dans l'alcool amylique, donne la réaction de Gmelin et rappelle la biliverdine et l'oocyane de Lemberg (1934). Pigment vert: bande diffuse de 712 à 680 m$\mu$.; pigment bleu: bande diffuse de 698 à 684 m$\mu$. (voir aussi Griffiths, 1892*b*).

## (3) *Dérivés de la chlorophylle*

La formule XXI montre la structure de la chlorophylle *a* d'après Fischer & Wenderoth (1939). Le squelette pyrrolique de la chlorophylle contient un noyau isocyclique et deux atomes d'hydrogène de plus que les porphyrines. Les deux H se trouvent dans le noyau IV et sont la cause de l'activité optique des chlorophylles et de leurs dérivés. Les pigments obtenus à partir des chlorophylles par ouverture

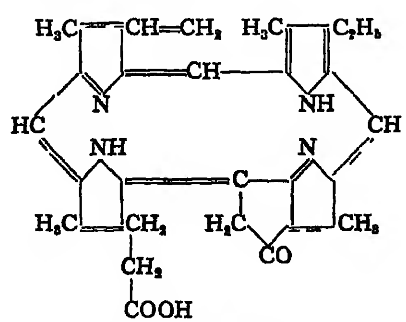



XXI. Chlorophylle *a*

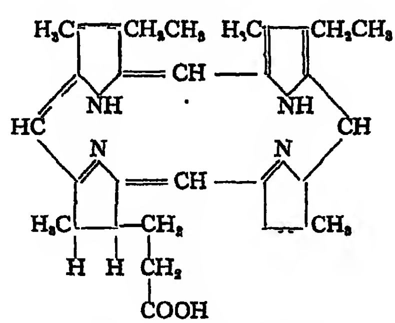
du noyau isocyclique ont un squelette porphyrique avec les mêmes deux atomes d'hydrogène en plus: on les appelle *chlorines* (XXIII).

(*a*) *Dégradation biologique de la chlorophylle.*

Chez les Vertébrés, la phylloérythrine $C_{33}H_{34}O_3N_4$ est le produit principal de dégradation de la chlorophylle *a* par l'intestin. Elle se forme par enlèvement du magnésium, saponification du phytol et de l'alcool méthylique et décarboxylation au carbone 10 (Fischer & Hilmer, 1925).




XXII. Phylloérythrine




XXIII. Mésopyrrochlorine

Chez les Vers à soie la dégradation va plus lion, avec scission de l'anneau isocyclique et formation de *phyllobombicine*, isolée par Fischer & Hendschel (1931) à partir des fèces de ce Ver. Ce pigment, $C_{34}H_{36}O_6N_4$, est transformé en chlorine par action d'alcoolate, en phylloporphyrine par l'acide bromhydrique et en phéoporphyrine par l'acide iodhydrique. Fischer & Hendschel ont isolé le même pigment à partir des fèces de la chenille d'*Acherontia atropos*. Seybold & Egle (1938) croient que la phyllobombicine n'est qu'un produit artificiel formé au cours de l'isolement; dans des fèces frais ils n'ont trouvé que les chlorophylles *a* et *b*.

La phylloérythrine, ainsi que la phyllobombicine, sont dérivés de la chlorophylle *a*; on ne connaît pas de produits de dégradation biologique de la chlorophylle *b*. Dans les fèces du mouton, Fischer & Hendschel (1932) ont trouvé deux autres dérivés de la chlorophylle *a*, les "probophorbides" *a* et *b*. Ces pigments se trouvent dans les excréments de nombreux autres Vertébrés (voir aussi Kohler *et al.* 1939).

(*b*) *Pigment verts, tégumentaires et autres.*

Les colorations vertes des téguments des Invertébrés sont dues en général à la présence d'algues symbiotiques, à la chlorophylle vraie ou à d'autres pigments verts dérivés de celle-ci. (En ce qui concerne les colorations vertes dues aux pigments biliaires, voir le chapitre précédent.)

*Coloration verte due à la présence d'algues symbiotiques.* Fürth (1903) a fait l'historique de la "chlorophylle animale" des Polypes, de nombreux Infusoires, Turbellaires, Planaires et autres. Entz (1876), puis Brandt (1883), ont trouvé que cette coloration est uniquement due à la présence d'algues microscopiques ("zoochlorelles").

La *marénnine*, pigment vert des Huîtres de Marenne, colorant les branchies et les palpes labiaux de divers Lamellibranches est due à une diatomée, *Navicula ostrearia* (Chatin & Muntz, 1894; Sauvageau, 1907. La nature chimique de la marénnine n'est pas encore connue, en raison surtout de difficultés d'extraction sans décomposition. D'après Ranson (1937) il s'agit d'un carotiprotéde.

*Coloration verte due à la présence de chlorophylle.* La chlorophylle alimentaire est surtout utilisée par les Insectes pour la coloration des téguments; il y a ainsi adaptation à la coloration de l'environnement. On trouve la chlorophylle (identifiée par des observations spectroscopiques) dans les élytres des Cantharides (MacMunn, 1882–3), dans les ailes des Phyllies (Becquerel & Brogniart, 1894), dans les ailes de *Locusta* (Podiapolski, 1907) et dans le tégument du Dixippe (Przibram & Lederer, 1933). Vegezzi (1916) a identifié à l'aide de l'adsorption chromatographique l'hépatochlorophylle des Mollusques avec les chlorophylles végétales.

Heilbron *et al.* (1935) ont identifié avec la chlorophylle *a* le pigment vert des tentacules d'*Anemonia sulcata.*

La chétoptérine est le pigment vert foncé de l'épithélium intestinal du Chétoptère (Annélide polychète tubicole). Romieu (1922) en a obtenu des cristaux. D'après son spectre d'absorption (655, 600, 535, 500 m$\mu$. dans l'alcool neutre) il pourrait s'agir d'une phéophytine ou phéophorbide, c'est-à-dire du produit résultant de l'élimination du magnésium ou du magnésium et du phytol des chlorophylles.

*Coloration verte due à la présence de pigments spéciaux.* Nous décrivons ici plusieurs pigments tégumentaires de Vers.

*Bonelline.* La femelle du Géphyrien *Bonellia viridis* est colorée en vert foncé. Son pigment, la bonelline, a intéressé beaucoup de chercheurs; Dhéré & Fontaine (1932) en citent une vingtaine. Sorby (1875) fut le premier à étudier son spectre d'absorption qui présente six bandes distinctes la différenciant nettement de la chlorophylle. Dhéré & Fontaine (1932), dans une étude spectrochimique très approfondie, concluent que la bonelline présente beaucoup d'analogies avec les porphyrines et la phylloérythrine et semble dériver des chlorophylles alimentaires.

Nous avons récemment obtenu la bonelline à l'état cristallisé après purification par adsorption chromatographique (Lederer, 1939*b*). Elle cristallise sous forme de fines aiguilles vert foncés, qui ne fondent pas jusqu'à 300°. Elle est soluble dans l'éther éthylique neutre, et dans les acides et les bases aqueuses, insoluble dans

l'éther de pétrole. Son spectre d'absorption se rapproche de très près de celui de la mésopyrrochlorine ($C_{31}H_{36}O_2N_4$) de Fischer *et al.* (1936) (XXIII):

| | |
|---|---|
| Mésopyrrochlorine dans le dioxane | 640, 612, 588, 541, 518, 492, 484 m$\mu$. |
| Bonelline dans le dioxane | 638, 608, 585, 537, 516, 492, 481 m$\mu$. |
| Mésopyrrochlorine dans HCl | 634, 590, (538), 520 m$\mu$. |
| Bonelline dans HCl | 633, 585, (536), 519 m$\mu$. |

D'après Stern & Molvig (1937) la bande à 610 m$\mu$. est caractéristique pour le groupe méso en position 2; le dédoublement de la sixième bande en deux bandes très rapprochées et de même intensité est très caractéristique pour ces chlorines et s'observe également pour la bonelline.

La bonelline serait donc une mésochlorine naturelle (peut-être une dioxy-méso-pyrrochlorine); sa formule brute $C_{31}H_{36}O_4N_4$ ainsi que sa capacité de former des sels complexes s'accordent avec cette hypothèse.

Pour Dhéré & Fontaine (1932) le rôle de la bonelline réside dans ses propriétés de photosensibilisateur; pour Michel (1931) la bonelline peut intervenir comme moyen chimique de défense. Dubois (1907) a observé que la Bonellie, exposée au soleil, s'entoure d'un nuage vert de bonelline.

*Phyllodocine.* Le pigment vert du tégument de *Phyllodoce viridis* ou *P. laminosa* (Annélide polychète), nommé phyllodocine par MacMunn (1889), ne présente pas de bandes d'absorption caractéristiques. Abeloos & Teissier (1926) disent que ce pigment, en grains figurés, est soluble dans l'eau et dans l'alcool, un peu soluble dans l'éther. Il est vert en milieu acide, rouge en milieu alcalin.

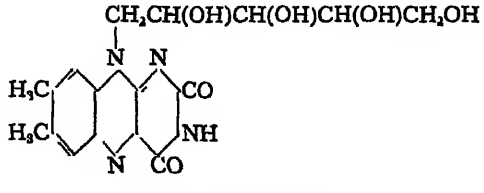
*Pigments d'*Eulalia viridis. MacMunn (1889) avait cru le pigment tégumentaire d'*Eulalia viridis* (Annélide polychète) identique à la phyllodocine. Ceci ne peut être exacte, d'après nos observations. Le pigment est soluble dans l'éther et passe dans le bicarbonate avec une couleur brune. La phyllodocine, au contraire, ne passe pas dans le bicarbonate, ni dans la soude. Repris par l'éther et filtré sur une colonne de chaux, le pigment d'*Eulalia* donne deux anneaux bruns. Il y a en outre un pigment vert, insoluble dans le bicarbonate, mais soluble dans la soude avec une couleur violette. Aucun de ces pigments ne possède une fluorescence.

*Pigments de* Thalassema lankesteri, Hamingia arctica *et* Aeolosoma tenebrarum. Deux Géphyriens, voisins de la Bonellie, ont également la peau colorée en un vert intense. C'est *Thalassema lankesteri* et *Hamingia arctica* dont le pigment serait le même, d'après Ray-Lankester (1898). La "thalassémine" n'a pas de fluorescence et ne possède qu'une seule bande d'absorption (vers 617 m$\mu$.). Elle est donc entièrement différente de la bonelline (Newbigin, 1898).

Le tégument de l'Oligochète *Aeolosoma tenebrarum* contient des gouttes d'huile colorées en vert. Le pigment est soluble dans les acides faibles; virage au rouge pourpre par addition d'alcali; pas de bande d'absorption (Beddard 1889; Griffiths, 1898).

## V. FLAVINES

A l'exception de l'aquoflavine de Koschara (1936), toutes les flavines naturelles se sont avérées identiques à la lactoflavine, $C_{17}H_{20}O_6N_4$ (XXIV). (Pour la chimie de la lactoflavine voir Kuhn, 1935, Karrer, 1939.)



XXIV. Lactoflavine

Le rôle biologique de ce pigment est basé essentiellement sur sa participation aux phénomènes d'oxydo-réduction de la cellule. La lactoflavine exerce le plus souvent son action comme groupe prosthétique de "ferments jaunes" qui effectuent la déshydrogénation de l'acide hexosephosphorique, la désamination de l'alanine, la déshydrogénation de la xanthine, etc. Dans ces groupes prosthétiques, la lactoflavine est liée à l'acide phosphorique et, par son intermédiaire, à des bases puriques ou pyridiniques. (Voir Bigwood, 1935; Fontaine, 1938; Warburg & Christian, 1938; pour le dosage, voir Gourévitch, 1937; et Lunde, Kringstad & Olsen, 1939.)

Il est probable que la plupart des cellules vivantes ont besoin de flavines et en contiennent une certaine quantité. Les parasites vivant en anaérobiose ne contiennent pas plus de flavines que d'autres Invertébrés vivant à l'air libre.

Drilhon & Busnel (1939) ont trouvé que la flavine est concentrée chez les Insectes dans les tubes de Malpighi surtout. La teneur en flavine augmente au cours du développement. Dans les crypto-néphridiens, la flavine se trouve le plus souvent liée à des protéines et accompagnée d'un corps à fluorescence bleue (voir Gourévitch, 1937). C'est chez les Insectes se nourrissant de végétaux frais que la teneur en flavine des tubes de Malpighi est la plus élevée. Les flavines jouent-elles un rôle dans ces organes, ou sont-elles seulement déposées comme produits inutiles ou destinées à être excrétées? On ne le sait pas; mais on peut croire qu'elles prennent part au métabolisme azote intense de ces glandes.

La lactoflavine joue aussi chez les Insectes le rôle d'une vitamine (Trager & Sabbrarow, 1938). Les larves des Moustiques de la fièvre jaune ont, en effet, besoin d'un apport de flavine (et de vitamine $B_1$) pour se développer normalement; une métamorphose normale n'est assurée qu'en présence d'un complexe de flavine et purine.

Bierry & Gouzon (1937) ont mesuré le spectre de fluorescence d'un pigment d'*Holothuria forskali* qui est jaune et a une forte fluorescence verte (axe à 532 m$\mu$.). Ce corps n'est pas une flavine. Il est très sensible à l'action des bases et des acides; Krukenberg (1883) le compte parmi les "uranidines", groupe de pigments prémélaniques. Nous avons bien pu confirmer les données de Bierry & Gouzon, mais pas celles de Krukenberg.

## VI. PTÉRINES

Les ptérines sont répandues surtout chez les Insectes; elles constituent le pigment jaune des Guêpes et les colorations blanches, jaunes et rouges de beaucoup de Papillons, surtout des Piérides (Hopkins, 1889–94; Wieland & Schöpf, 1925; Schöpf & Becker, 1933). Une ptérine (l'uroptérine) se trouve aussi dans l'urine et dans différents organes des Vertébrés (Koschara, 1936). Ce pigment se trouve également dans l'herbe et serait synthétisé par l'organisme humain (Koschara & Haug, 1939).

Les ptérines sont des dérivés de la purine et contiennent de 30 à 40 % d'azote. Ce sont des composés plus ou moins hydrosolubles, et insolubles dans les solvants organiques. Elles sont amphotères, plus solubles dans les acides et les bases faibles que dans l'eau pure. Quelques-unes cristallisent bien, d'autres n'ont été obtenues que sous forme de dérivés cristallisés ou d'amas microcristallins. Leur point de fusion n'est pas bien défini et on n'est pas parvenu à en préparer des dérivés caractéristiques. Ainsi l'identification de ces corps est fort difficile; il faut souvent se contenter de les caractériser par leur teneur en azote, leur spectre d'absorption, leur fluorescence et leur comportement envers des adsorbants (Schöpf & Becker, 1936). Il est même plus prudent de considérer des termes comme "xanthoptérine" ou "érythroptérine" plutôt comme désignation d'un groupe de corps très voisins que comme nom d'un pigment pur.

Hopkins (1889–92) a été le premier à isoler le pigment blanc des ailes du Piéride du chou (*Pieris brassicae*), et la xanthoptérine, pigment jaune des ailes de *Gonepteryx rhamni* L. Il a cru que le premier n'était autre que de l'acide urique, et a montré que le deuxième en est un dérivé. Schöpf & Wieland (1926) ont trouvé que le premier, la leucoptérine, n'est pas l'acide urique, mais bien un pigment défini. Wieland & Schöpf (1925), puis Schöpf & Becker (1933) ont étudié la xanthoptérine qu'ils ont découvert également dans le tégument des guêpes, et Schöpf & Becker (1936) ont décrit plusieurs nouvelles ptérines, dont l'érythroptérine, pigment rouge, est le plus important. Becker & Schöpf (1936) ont élaboré des méthodes microchimiques permettant d'isoler et de distinguer les différentes ptérines. En même temps, Koschara (1936) isole l'uroptérine de l'urine humaine, pigment jaune probablement identique à la xanthoptérine.

On ne sait rien de précis sur le rôle physiologique des ptérines chez les Insectes. Becker (1937) a trouvé que ces pigments ne sont pas excrétés par les tubes de Malpighi et ne peuvent donc pas être considérés comme produits d'excrétion proprement dit.

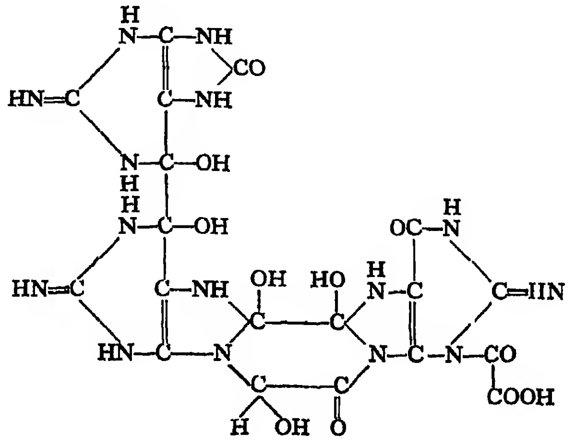
Plusieurs mémoires traitent du rôle de l'uroptérine chez les Mammifères (Wolf & Tschesche, 1937; Koschara & Hrubesch, 1939; Koschara & Haug, 1939). L'uroptérine jouerait un rôle de donateur d'hydrogène dans le métabolisme oxydatif des amino-acides et des purines. Ce rôle paraît d'autant plus vraisemblable que l'uroptérine est réduite de façon réversible par le glutathion (Koschara, 1936).

*La leucoptérine* ($C_{19}H_{19}O_{11}N_{15}$ ou $C_{19}H_{17}O_{11}N_{15}$) a d'après Wieland *et al.* (1933) une des formules XXV. La leucoptérine donne la réaction de murexide, elle fournit trois molécules d'acide oxalique dérivées des ponts réunissant les trois

noyaux puriques. L'hydrolyse par l'acide chlorhydrique à 170° donne trois molécules d'ammoniaque de plus que l'hydrolyse de l'acide urique. Chaque noyau purique de la leucoptérine réagit avec le chlore et l'alcool avec formation d'un éther glycolique comme l'acide urique. Fromherz & Kotzschmar (1938) se prononcent plutôt pour la formule A qui correspondrait mieux au spectre d'absorption de la leucoptérine; mais ils pensent que les noyaux sont plus fortement liés les uns aux autres.

On prépare la leucoptérine par extraction ammoniacale des ailes dégraissées de *Pieris brassicae*. L'extrait, évaporé à un petit volume, dépose la leucoptérine amorphe que l'on obtient sous forme de cristaux après dissolution répétée dans la soude et

A



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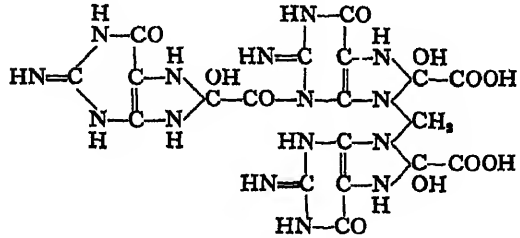

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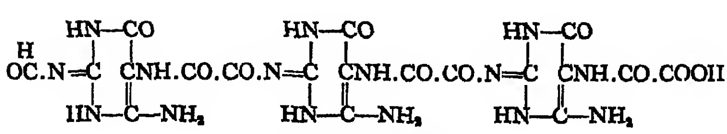


XXV. Leucoptérine

précipitation par de l'acide (215,000 papillons ont fourni 1164 gr. d'ailes, 40 gr. de produits bruts et 16 gr. de leucoptérine cristallisée).

La leucoptérine donne un sel disodique jaune, bien cristallisé. Pour la préparation de différents dérivés et une étude détaillée de ses réactions voir Wieland *et al.* (1933), ainsi que Wieland & Kotzschmar (1937).

La leucoptérine est accompagnée dans les extraits des ailes de *Pieris brassicae* d'une anhydroleucoptérine contenant trois molécules d'eau de moins.

Schöpf & Becker (1933) ont trouvé la leucoptérine dans les ailes de *Gonepteryx rhamni* et dans les parties blanches des ailes de *Euchloe cardamines*. Les sels de sodium de leucoptérine de différentes provenances ont une teneur en sodium variant de 6, 2 à 9, 4 %. Schöpf & Becker pensent qu'il existe au moins deux leucoptérines isomères d'acidité différente; ils appellent leucoptérine *a*, celle dont le sel de sodium contient le moins de sodium, leucoptérine *b*, l'isomère dont le sel de sodium a la plus forte teneur en sodium.

*Guanoptérine*. Ce corps, $C_{19}H_{20}O_3N_{20}$ ou $C_{19}H_{22}O_4N_{20}$, trouvé par Schöpf & Becker (1936) dans les ailes de *Appias nero*, *Catopsilia argante*, *Euchloe cardamines* et *Gonepteryx rhamni*, est également incolore et cristallise sous forme de fines aiguilles. Son sulfate est peu soluble et cristallise bien. La guanoptérine ressemble à la guanine, comme la leucoptérine ressemble à l'acide urique. Elle en diffère par sa grande tendance à cristalliser, par sa solubilité dans l'ammoniaque et par sa plus grande teneur en azote. Comme la guanine, elle ne donne pas la réaction de murexide. La réaction de Weidel au chlorate est positive comme pour la guanine. La guanine n'a pas de fluorescence. Elle contiendrait d'après Schöpf, Becker, et Reichert trois ou quatre groupements de 2:5 diamino-pyrimidine, XXVII; dans *Catopsilia argante*, *C. rurina* et *C. statira*, la guanoptérine est accompagnée de xanthine (Purrmann, 1939).

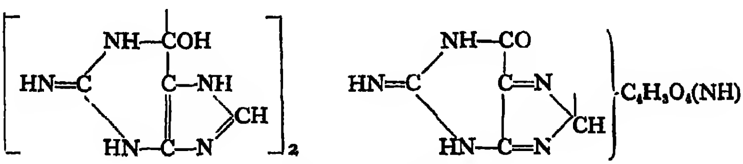
*Mésoptérine*. Les propriétés (couleur, solubilité, teneur en C et N, 39 % de C, 3 % de H, 36 % de N) de ce corps faiblement basique, le placent entre la leucoptérine et la xanthoptérine. Schöpf & Becker (1936) l'ont isolé sous forme de cristaux presque incolores à partir des ailes des femelles de *Gonepteryx rhamni* et du tégument de *Vespa germanica*.

*Xanthoptérine*. On peut obtenir ce pigment jaune ($C_{19}H_{18}O_6N_{16}$) à l'état pur sous forme de son sel de barium $C_{19}H_{14}O_7N_{16}(Ba/2)_6$ dérivé d'un hydrate. Par décomposition de ce sel on obtient différents hydrates de la xanthoptérine également cristallisés. La xanthoptérine donne la réaction de murexide. Son spectre d'absorption présente deux faibles bandes vers 370 et 390 m$\mu$. (dans l'acide acétique glacial) et une forte bande vers 280 m$\mu$. correspondant aux noyaux pyrimidiniques.

L'identification de la xanthoptérine est facilitée par sa fluorescence (jaune en solution faiblement acétique, bleu-clair en solution neutre et bleu-vert en solution carbonatée) et son comportement envers des adsorbants (Becker & Schöpf, 1936; Koschara, 1936). Une solution de xanthoptérine dans le méthanol contenant 0·01 % d'HCl, donne sur l'alumine une zone jaune ayant une fluorescence jaune-vert intense en lumière ultra-violette. La longueur de cette zone est à peu près proportionnelle à la quantité de la xanthoptérine. On ne peut plus éluer le pigment; il est décomposé dans l'adsorbat. La xanthoptérine est également adsorbée sur la franconite et peut en être éluée sans destruction.

La présence de la xanthoptérine à côté de la leucoptérine et sa composition analogue indiquaient déjà une parenté étroite entre deux corps. Cette parenté a été prouvée en 1939 par Wieland & Purrmann. L'oxydation de la xanthoptérine par l'eau oxygénée donne un corps incolore cristallisé, $C_{19}H_{20}O_{10}N_{16}$, dont les propriétés et la formule brute rappellent la leucoptérine. C'est en effet une imino-leucoptérine, c'est-à-dire une leucoptérine dans laquelle à un atome d'oxygène se substitue le

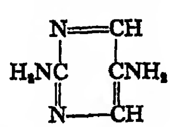
groupement —NH. L'action de l'acide nitreux remplace dans la leucoptérine 3C=NH par 3C=O. L'imino-leucoptérine donne la même réaction avec quatre groupes C=NH; dans les deux cas, le produit de réaction, la desimino-leucoptérine est identique. Le squelette des deux ptérines est donc le même; dans la xanthoptérine, un groupe NH remplace un atome d'oxygène de la leucoptérine. Si on l'enlève par hydrolyse, on obtient un corps $C_{19}H_{19}O_8N_{15}$, contenant trois atomes d'oxygène de moins que la leucoptérine. L'oxydation par $H_2O_2$ introduit ces trois atomes; ceci correspond à la transformation de trois molécules de xanthine en acide urique. La xanthoptérine serait ainsi un dérivé de la xanthine, comme la leucoptérine est un dérivé de l'acide urique (XXVI). Reste à élucider comment les trois noyaux de purine sont attachés les uns aux autres.



XXVI. Xanthoptérine

Schöpf & Kottler (1939) ont étudié l'action de l'acide chlorique sur la xanthoptérine et ont obtenu trois molécules d'oxalyl-guanidine HOOC—CO—NH—C(=NH)—$NH_2$ provenant des trois noyaux de pyrimidine de ce pigment.

Les résultats de l'hydrolyse par l'acide chlorhydrique amènent Schöpf, Becker & Reichert (1939) à supposer la présence dans la xanthoptérine de deux noyaux de 2:5 diamino-pyrimidine (XXVII) et d'un noyau à structure quinoïde (XXIX).



XXVII. 2:5 Diamino-pyrimidine

D'après Schöpf, Kottler & Reichert (1939), la xanthoptérine des Piérides serait liée à l'allantoine sous forme d'un complexe hydrolysable à la baryte. Un exemplaire mâle de *Catopsilia rurina* contiendrait 0·33 mg. d'allantoine pour 1·3 à 2·4 mg. de xanthoptérine.

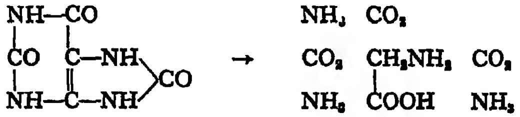
*Chrysoptérine.* La composition chimique et quelques propriétés de ce pigment orange ($C_{19}H_{16}O_8N_{14}$), isolé par Schöpf & Becker (1936) à partir de *Gonepteryx rhamni*, le placent entre la xanthoptérine et l'érythroptérine. Il pourrait être une xanthoptérine dans laquelle deux groupes NH seraient remplacés par 2O; ceci expliquerait sa plus grande acidité. Ses solutions dans l'acide acétique *n*/2 et l'HCl *n*/250 ont une fluorescence violet-bleue comme l'érythroptérine. Elle se fixe plus fortement que la xanthoptérine sur l'alumine et, adsorbée, présente une fluorescence vert-jaune comme celle-ci. Le sel de barium de la chrysoptérine est amorphe, brun-jaune et plus soluble que celui de la xanthoptérine.

*Erythroptérine.* Ce pigment est la plus foncée des ptérines et se trouve dans les parties rouges des ailes de plusieurs Piérides (*Appias nero*, *Euchloe cardamines*, etc.). Des colorations orangées, comme celles des mâles de *Catopsilia argante* et *Colias edusa*, sont dues à des mélanges d'érythroptérine et de xanthoptérine (Schöpf & Becker, 1936).

On prépare l'érythroptérine par extraction ammoniacale et précipitation par l'acide chlorhydrique. On purifie le produit brut par dissolution et précipitation répétée et obtient ainsi des préparations microcristallines contenant de 30 à 33 % d'azote. L'érythroptérine est caractérisée par sa couleur rouge (bandes d'absorption vers 450, 420 et 300 m$\mu$.), par sa teneur en azote relativement faible, par sa fluorescence violet-bleue (dans l'acide acétique $n/2$) et par son adsorbabilité. En solution faiblement acide, elle se fixe sur l'alumine plus fortement que la xanthoptérine, en une zone mince ayant une fluorescence brune; cependant l'adsorption détruit le pigment. L'érythroptérine se transforme facilement en un produit violet, amorphe, peu soluble dans l'ammoniaque et mieux adsorbé qu'elle-même.

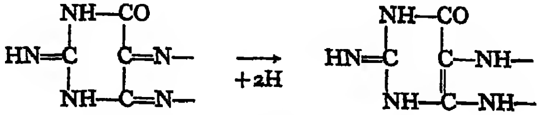
La composition élémentaire, ainsi que la position des bandes d'absorption, varient pour différentes préparations d'érythroptérine; il existe probablement plusieurs érythroptérines peu différentes l'une de l'autre. La formule de ces corps est à peu près $C_{19}H_{17-18}O_{8-10}N_{13-11}$; la somme des atomes d'azote et d'oxygène doit être de 20 ou 21.

Schöpf, Becker & Reichert (1939) ont établi des rapports chimiques étroits entre l'érythroptérine et les autres ptérines en étudiant la scission par l'acide chlorhydrique. L'acide urique et la xanthine donnent chacune plusieurs molécules d'ammoniaque, de gaz carbonique et une molécule de glycine (XXVIII). La



XXVIII. Hydrolyse de l'acide urique

guanoptérine donne quatre molécules de glycine, la leucoptérine trois, la xanthoptérine deux, et l'érythroptérine une seule. Ceci établi, Schöpf, Becker & Reichert ont pensé que la xanthoptérine et l'érythroptérine pourraient contenir en outre des noyaux fournissant de la glycine, d'autres noyaux isomères de structure quinoïde



XXIX. Forme quinoïde et sa réduction

(XXIX) ne fournissant pas de glycine à l'hydrolyse. Cette structure expliquerait en outre la couleur de ces ptérines. Par réduction on devrait pouvoir les transformer en noyaux "normaux" donnant de la glycine. L'expérience a vérifié cette hypothèse. La xanthoptérine et l'érythroptérine sont décolorées par l'action de zinc et de l'acide

formique et les produits de réduction fournissent alors trois molécules de glycine à l'hydrolyse. La xanthoptérine contient donc deux noyaux de 2:5 diamino-pyrimidine normaux et un de structure quinoïde (couleur jaune); l'érythroptérine contient un noyau normal et deux noyaux quinoïdes conjugués (couleur rouge).

*Autres ptérines.* Becker & Schöpf (1936) ont trouvé deux ptérines, l'une incolore, amorphe, l'autre cristallisable, dans les ailes du Neuroptère *Ascalaphus libelloides*. La première donne une forte réaction de murexide et a une vive fluorescence bleu-violette. Elle diffère de la leucoptérine par son sel de sodium incolore et par son fort pouvoir réducteur; la deuxième a un sel de sodium également incolore et contient 48·3 % d'azote. Un pigment analogue, mais non identique, se trouve dans les taches blanches de l'hypoderme de *Lasiopticus pyrastri*. Dans *Catopsilia argante*, une "ptérine acide" accompagne la xanthoptérine.

*Pigments des yeux de* Drosophila melanogaster. La coloration des yeux de *Drosophila melanogaster* qui joue un grand rôle dans les travaux des généticiens, est due, d'après Schultz (1935), à deux pigments hydrosolubles, l'un jaune, l'autre rouge. Nous avons purifié le pigment rouge d'une souche sauvage; il semble qu'il ait une parenté avec les ptérines. Cette "drosoptérine" est plus foncée encore que l'érythroptérine (bande d'absorption à 465 mμ. dans l'eau); elle n'a pas de tendance à cristalliser. Purifiée par précipitation sous forme d'un sel d'argent, par extraction au phénol et par les méthodes de Schöpf & Becker, elle se présente sous forme d'une poudre, rouge foncée, ayant 42 % de C, 5·5 % de H et environ 19 % de N. Elle est accompagnée d'un corps incolore à forte fluorescence bleue rappelant celui observé par Schöpf, Kottler & Reichert (1939).

Rappelons à ce sujet les travaux sur une hormone qui provoque la formation de pigment d'yeux chez la Drosophile et chez *Ephestia* (Khouvine *et al.* 1936; Khouvine & Ephrussi, 1937; Khouvine *et al.* 1938; Becker, 1938).

## VII. MÉLANINES

Les mélanines, très répandues chez les Invertébrés, ont résisté jusqu'ici à une étude chimique approfondie. L'intérêt principal se concentre ici sur leur formation et sur les produits intermédiaires.

*Formation des mélanines.* On est d'accord, en général, pour considérer la mélanogénèse comme un phénomène enzymatique, dû à un ou plusieurs ferments. Le plus répandu, la tyrosinase, est d'après Dalton & Nelson (1938), une protéine cristallisable contenant du cuivre (voir aussi Kubowitz, 1938*a*, *b*). Les rapports entre tyrosinase, catéchol-oxydase et déshydrogénases sont encore assujettis à des discussions (Califano & Kertesz, 1938) et dépassent le cadre de la présente revue.

Le mécanisme de la mélanogénèse (XXX) a été établi par Raper (1927, voir aussi Dulière & Raper, 1930).

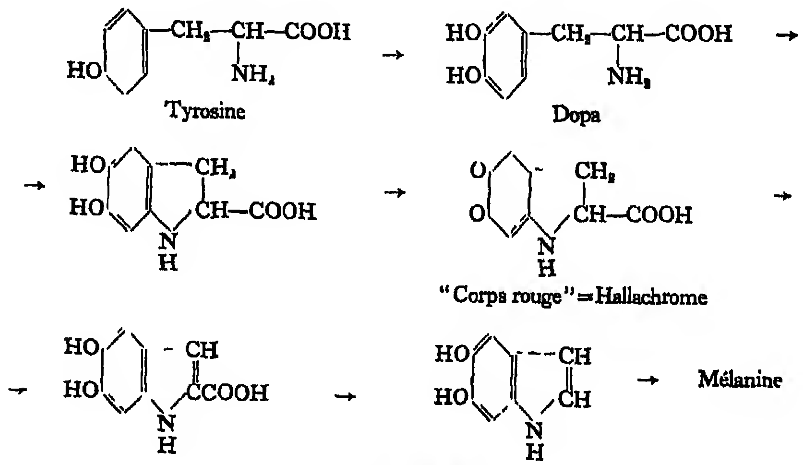
Pour une revue des nombreux travaux biologiques sur la formation de la mélanine chez les Invertébrés, et la mélanogénèse non-fermentaire voir Verne (1926).

*Produits intermédiaires.* Le produit intermédiaire rouge (XXX) est identique à l'*hallachrome*, pigment du Ver polychète, *Halla parthenopaea*, isolé par Mazza &

Stolfi (1931). Ce pigment est rouge à un $pH$ inférieur à 8·3 et vert à un $pH$ plus alcalin. La forme rouge est soluble dans l'alcool amylique, la forme verte, dans l'eau seulement. Ce pigment peut être réduit et oxydé réversiblement; il augmente de plusieurs centaines de % la respiration des œufs d'Oursins non-fertilisés et des œufs d'*Ascaris* fertilisés (Friedheim, 1933).

Un pigment analogue, dérivé de l'adrénaline, est l'*adrénochrome* (Green & Richter, 1937). Ce corps se transforme *in vitro* facilement en mélanine; il est possible que ce phénomène ait aussi une importance *in vivo* (Stefl, 1931).

Citons encore un *pigment violet dans la rétine de Céphalopodes* trouvé par Krukenberg (1878) et étudié récemment par Escher-Desrivières *et al.* (1938). Par sa localisation dans les franges de l'épithélium pigmentaire, par ses caractères morphologiques et ses migrations le long des franges de l'épithélium, il se rapproche des mélanines rétiniennes des Vertébrés. Il en diffère cependant par sa couleur et sa solubilité dans les alcalis faibles. Il diffère aussi du pourpre rétinien




XXX. Mélanogénèse

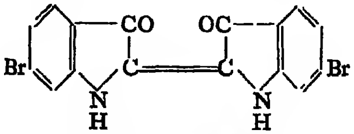
des Vertébrés par sa stabilité à la lumière et par l'absence de vitamine A. Le pigment a le caractère d'un chromoprotéide. Le groupe prosthétique est également violet et contient 8 % d'azote.

Verne (1923) a étudié chez les Crustacés Décapodes un "*pigment amino-acide*". Il forme sur l'animal des granulations variant du blanc sale au jaune plus ou moins foncé et capable de se transformer en mélanine.

Il faut également mentionner ici les "*Uranidines*" de Krukenberg, pigments jaunes de plusieurs Invertébrés (Corails jaunes, Éponges jaunes, Holothuries, Annélides, Ascidies, etc.) qui se transformeraient en pigments mélaniques. Nous pensons que les extraits d'uranidines contenaient un mélange de pigments dont la plupart n'avaient que peu de rapport avec des mélanines. Prenant (1922) attribue une structure quinonique à certains de ces pigments, trouvés dans le tégument de quelques Prosobranches (*Littorina*, *Calyptraea*) et Annélides (*Serpula*, *Polydora*, *Syllis*).

## VIII. PIGMENTS DIVERS

*Pourpre des Mollusques.* Les Gastéropodes *Purpura haemastoma*, *P. papillus*, *Murex brandaris*, *M. trunculus* et *M. erinaceus* ont servi dans l'antiquité à la fabrication de la pourpre. Ce pigment est sécrété à l'état de chromogène incolore par la glande à pourpre qui se rattache aux glandes hypobranchiales. L'irradiation du chromogène produit une coloration verte, qui vire au bleu, puis au rouge-pourpre. Pour Dubois (1909) un ferment (purpurase) joue un rôle dans la genèse du pigment à partir du chromogène. Friedländer (1909) a obtenu la pourpre à l'état cristallisé, et en a effectué la synthèse. C'est un 6:6′-dibromo-indigo (XXXI).



XXXI. Pourpre antique

*Pigments indicateurs de p*H. Plusieurs auteurs ont décrit des pigments d'Invertébrés caractérisés par des virages de couleur à différents *p*H. Leur constitution chimique étant inconnue, nous les réunissons ici dans un même chapitre. Rappelons toutefois que plusieurs pigments dont nous avons parlé plus haut, changent de couleur à différents *p*H.

Moseley (1877) a trouvé chez un Planaire terrestre (*Rhynchodemus*) un pigment bleu qui vire au rouge en milieu acide; plusieurs autres pigments intéressants colorent les Vers; le mémoire de Dhéré & Fontaine (1932) en contient une énumération complète.

Crozier (1916*a*, *b*, 1918) étudie la perméabilité de différents acides dans les cellules vivantes du Nudibranche *Chromodoris zebra* dont le pigment bleu vire au rouge à un *p*H de 5·6. Il étudie aussi les pigments indicateurs de *Velella*, *Melita* et *Stichopus*.

Abeloos & Teissier (1926) décrivent le pigment d'*Antedon bifida*, qui existe dans les pinnules sous deux formes: granulations rouges ou mottes d'un jaune verdâtre. Le pigment rouge est soluble dans l'eau, insoluble dans l'éther. Sous l'influence des bases, la coloration vire au violet et le pigment précipite. Sous l'action des acides la coloration passe au jaune. Si le pigment de la variété jaune présente les mêmes caractères de solubilité que le pigment rouge, il n'offre pas de changements de couleur à différents *p*H. Mais soumis à l'ébullition avec un alcali, il se transforme en un pigment qui présente toutes les variations de coloration du pigment rouge, et semble lui être identique.

Le pigment verdâtre du pied d'*Haliotis tuberculata* est, d'après Abeloos & Teissier (1926), soluble dans l'alcool, insoluble dans l'éther; il est jaune verdâtre en milieu acide, rouge en milieu alcalin. On peut le rapprocher, peut-être, des pigments biliaires, ainsi que le pigment de la tunique de *Styelopsis grossularia*, qui est rouge à l'état naturel, soluble dans l'eau et dans l'alcool. Ses solutions sont vertes en milieu acide, rouges en milieu alcalin (Abeloos & Teissier, 1926).

Payne (1930–1) a résumé les travaux ayant trait aux pigments des Hydraires et décrit ceux des Sertulariidés. Plusieurs pigments jaunes et bruns, solubles dans l'eau et l'alcool, peu solubles dans l'éther, se décolorent à un *p*H inférieur à 6·5. *Sertularia argentea* contient un corps incolore en solution neutre ou acide, qui devient jaune à *p*H 8, orange à *p*H 8·5 et brun à *p*H 10. Le pigment de *Sertularia pumila* est brun à l'état alcalin et neutre, jaune à *p*H 6·5 et incolore à *p*H 4. Voir aussi Teissier & Volkonsky (1929) sur les pigments de l'Hydraire *Sertularella* et l'Éponge *Clathrina*.

Haurowitz & Waelsch (1926) ainsi que Kropp (1931) ont décrit le pigment bleu de *Velella spirans*, qui vire au jaune, puis rose et rouge après acidification.

Just (1939) mentionne un pigment de l'Échinoderme *Echinarachnius* qui est vert en solution alcaline et pourpre en solution acide.

## IX. RÉSUMÉ

1. *Caroténoïdes*. Ces pigments sont beaucoup moins stables qu'on ne le croyait il y a quelques années. Ils s'isomérisent spontanément, s'oxydent en milieu alcalin et se décomposent en milieu acide. Ils se trouvent surtout dans les glandes sexuelles et les œufs des Invertébrés et y jouent peut-être un rôle biologique important.

2. *Quinones*. Les pigments d'Oursins isolés récemment (échinochrome, spinochrome et autres) sont des oxy-naphtoquinones. L'échinochrome, sécrété par les œufs d'*Arbacia*, active les mouvements des spermatozoïdes et les attire vers l'œuf. Les naphtoquinones méritent un grand intérêt biochimique depuis que l'on sait qu'ils peuvent agir comme vitamine K (antihémorragique).

Les pigments des Aptères du genre *Coccus* (cochenille, kermès et acide laccaïnique) sont des dérivés de l'anthrachinone. Leur rôle biologique est inconnu.

3. *Pigments pyrroliques*. (*a*) *Porphyrines*. Il existe chez le Lamellibranche *Pteria radiata* une porphyrine caractéristique, la conchoporphyrine, qui est en quelque sorte un intermédiaire entre l'uroporphyrine et la protoporphyrine. Des porphyrines colorent en outre le tégument de quelques Vers.

(*b*) *Pigments biliaires*. Les Invertébrés contiennent souvent la biliverdine ou des pigments analogues. Nous décrivons en détail plusieurs pigments spécifiques appartenant plus ou moins étroitement aux pigments biliaires. Ce sont: la rufine du tégument de la limace rouge, *Arion rufus*, la rufescine de la coquille d'*Haliotis rufescens*, les pigments bleu-verts de la coquille d'*Haliotis californiensis*, la calliactine de l'Anémone *Sagartia parasitica*, les pigments violets de la sécrétion d'*Aplysia* et un pigment vert des ailes de Lépidoptères.

(*c*) *Dérivés de la chlorophylle*. Les colorations vertes des Invertébrés peuvent être dues soit à des algues symbiotiques, soit à la présence de chlorophylle alimentaire, soit à des pigments spécifiques résultant d'une transformation de la chlorophylle. Parmi ces derniers, la bonelline, pigment du Ver *Bonellia viridis* est le plus intéressant; c'est une mésochlorine naturelle. Plusieurs autres espèces de Vers contiennent des pigments tégumentaires verts peu étudiés.

4. *Flavines*. Pour les Insectes, la lactoflavine est une vitamine comme pour les Vertébrés. Elle s'y trouve accumulée surtout dans les tubes de Malpighi.

5. *Ptérines.* Ces pigments sont répandus surtout chez les Insectes. Les plus importants sont: la leucoptérine des ailes du Piéride du chou, la xanthoptérine des ailes de *Gonepteryx rhamni* et du tégument des guêpes, et l'érythroptérine des taches rouges des ailes d'*Euchloe cardamines* et autres. Ce sont des dérivés de la purine. Les ptérines ne sont pas des produits d'excrétion; elle prendraient part au métabolisme azoté.

6. *Mélanines.* Ces corps ne se prêtent que difficilement à l'étude chimique. Ils se forment par action de la tyrosinase, ferment contenant du cuivre. Le Ver *Halla parthenopaea* contient un pigment rouge, l'hallachrome, que l'on peut considérer comme stade intermédiaire de la formation de mélanines à partir de la tyrosine. Un autre produit intermédiaire serait le pigment violet de la rétine des Céphalopodes.

7. *Pigments divers.* (*a*) La pourpre de certains Gastéropodes est un dibromo-indigo; le pigment est sécrété sous forme d'un chromogène incolore qui se transforme en pigment par action de la lumière.

(*b*) Les *pigments indicateurs de p*H sont très répandus chez les Invertébrés. Ils appartiennent aux classes les plus diverses (phénols, quinones, dérivés pyrroliques et autres).

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# THE PRIME VARIABLES OF MEIOSIS

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Meiosis is a method of nuclear division leading to an orderly reduction of the chromosome number. It is coextensive with sexual reproduction and in the sexual cycle it compensates for fertilization. These statements provide us with a minimum definition of the process. Beyond this we know that it does not follow precisely the same course in any two species, or indeed, for many species, in any two of their cells. We also know that beneath this diversity there is a uniformity of form and action in meiosis which although less obvious is not less significant. This uniformity we have to understand first. It bears witness at once to a common origin in the evolution of the organism and to a common rule in the mechanics of the cell.

The assumption of this common origin and this common rule gives the name of meiosis a meaning and a use. It enables us to set about understanding the process and discovering indeed what the origin was and what the rule is. For this purpose we first have to establish a basis of comparison; we have to take a basic type of meiosis. The criterion of such a basic type is that it should occur more widely than any other; that indeed none of the great groups of plants, animals and protista should entirely escape from it. Using this type we may legitimately approach our two problems. First, to discover by the comparison of meiosis with a similar basic type of mitosis, from which it must be supposed to have arisen, how this evolutionary change took place. Secondly, to discover how the series of events that characterize meiosis can be arranged in a regular causal sequence.

Our basic type of mitosis is necessarily defined as a method of separating the pairs of chromatids, derived from earlier division of chromosomes, into two equivalent daughter nuclei by the division of their centromeres. Its universal character depends on bipolarity of reproduction, of attraction, and finally of separation. This principle is of course conditional, not absolute. Chromosomes are not bound to behave in this way in all cells of all organisms, nor in all conditions, natural or artificial. In cells off the beaten track of development we find indefinite reproduction, indefinite attraction and no separation. This leads in a simple form to the progeny pairing of diplochromosomes which can be imitated experimentally (Barber, 1940*a*), and in a specialized and adaptive form to the unlimited pairing of certain gland cells, which are more remote from our basic mitosis than is meiosis itself. They are more remote because, having no future, they are not limited by the requirements of genetic continuity which limit the conduct of meiosis and mitosis.

When we compare our two basic types of mitosis and meiosis we see that the difference between them arises at the earliest stage. The mitotic chromosomes are double. The meiotic chromosomes are single. This difference I was originally led to assume on the preconceived notion of a common rule of mechanics. It has now been tested and found consistent with X-ray analysis. From this single change the series of events in meiosis can be represented in sequence. Let me recall what this sequence is:

(1) Prophase begins before the division of the chromosomes; it is precocious.

(2) Homologous chromosomes, being single, attract one another specifically in pairs.

(3) Paired chromosomes, owing to internal torsion, twist round one another.

(4) Chromosomes divide, and in doing so upset their equilibrium in cohesion and attraction.

(5) The upset of cohesion leads to breakage, which releases the torsion and allows reunion of new pairs of ends, i.e. crossing-over.

(6) The breakdown of attraction leads to separation and the appearance of the points of crossing-over as chiasmata.

(7) The new attractions between pairs of threads, now chromatids, allow the chiasmata to hold pairs of non-attracting chromosomes together.

(8) Metaphase like prophase is precocious and comes before the centromeres can separately orientate and divide.

(9) Those centromeres which are held together by chiasmata between their chromosomes therefore co-orientate in pairs.

(10) The beginning of anaphase is determined therefore not by the division of the centromeres but by the lapse of chromatid attractions.

This causal sequence applies, so far as we know from combined cytological and genetical evidence, to one or both sexes in all sexually reproducing organisms. It resolves in terms of a single timing difference, or precocity, the three major contradictions of meiosis: first pairing at meiosis as against non-pairing at mitosis; secondly, co-orientation of centromeres at meiosis as against self-orientation at mitosis; and thirdly, attraction at early prophase against repulsion at late prophase, this last depending on the double function of crossing-over, which began by being physically necessary for the cell and is maintained by being adaptively desirable for the species.

This string of postulates or conjectures rests on a variety of evidences which have been separately reviewed elsewhere. They do not of course represent a final goal, a permanent statement of the truth about meiosis. Indeed they are important not as a record of achievement, but as a plan of attack. They seem to be the only proper assumptions to make in the light of our present knowledge, the assumptions therefore that have to be tested by prediction and experiment if any progress is to be made with this problem by planning as opposed to the more laborious method of general attrition.

The first line of this attack is to continue to apply natural tests. It is to find out what are the most widespread and characteristic variations affecting the causal

sequence. From these we shall find out what is inherent in it and what is capable of modification, what are in fact the prime variables of meiosis.

These variables are the ones that affect the first events in the sequence—before crossing-over takes place. They affect where pairing begins and how far it proceeds. In following their action it is convenient to distinguish between two arbitrary classes of chromosomes. They are those with the centromeres near one end (*S*) and those with them near the middle (*M*). The problem may be put in a nutshell by comparing the classical examples of two grasshoppers; *Stenobothrus*, in which chiasmata are found in all parts of the chromosomes, and *Mecostethus*, in which they are localized proximally, near the centromere. This difference follows from the earlier behaviour. In *Stenobothrus* we find that pairing begins at the ends in *M* and *S* without regard to the position of the centromere. In *Mecostethus* on the other hand, where all the chromosomes have their centromeres near one end, we find, first, that pairing always begins at this end, and secondly, that it is never complete (Fig. 1).

What is the cause of these two differences? Let us take first the incompleteness of pairing. Its explanation becomes clear when we compare meiosis in a series of species of *Fritillaria*. In these we find gradations between the two extremes of behaviour, with incomplete pairing and localized chiasmata on the one hand and with complete pairing and freely distributed chiasmata on the other. In the intermediate types there is considerable variation even within the same nucleus. The distal parts fail to pair, and fail in a varying degree. And even in species where pairing is usually complete the parts hampered in their movement by attachment to nucleoli sometimes fail to come together (Darlington, 1935*b*).

It seems then that in these species pairing begins near the centromere and is interrupted before it is complete. There is a *time limit* to attraction beyond which pairing ceases. It seems as though prophase had not begun early enough to allow the process of pairing to be completed before the chromosomes divide, as though, in other words, we were dealing with an intermediate condition between meiosis with pairing and mitosis without it.

Now in *Fritillaria* there are both *M* and *S* chromosomes and we should expect to find that the *M*'s having the regions which take the initiative in pairing near the middle of the chromosomes, and therefore tethered on both sides, would be slower in getting to work than the shorter *S* chromosomes with pairing regions near their ends. And where there is an early time limit they should achieve less pairing and fewer chiasmata than the *S* chromosomes. This is in fact the case in *F. Meleagris* and other species where localization is extreme (Fig. 5). We might also expect that where the time limit did not come too early, distal ends remote from the point of first pairing, being freer in movement than the middles, would sometimes come into contact and form chiasmata although the intercalary parts remained unpaired. Such intermittent or two-point pairing is in fact characteristic of the intermediate localization that we find in *Mecostethus grossus* as well as in *Fritillaria Elwesii*. We therefore find, first, that a gradation of behaviour within the individual shows that all parts of homologues are capable of pairing and crossing-over, and secondly, that

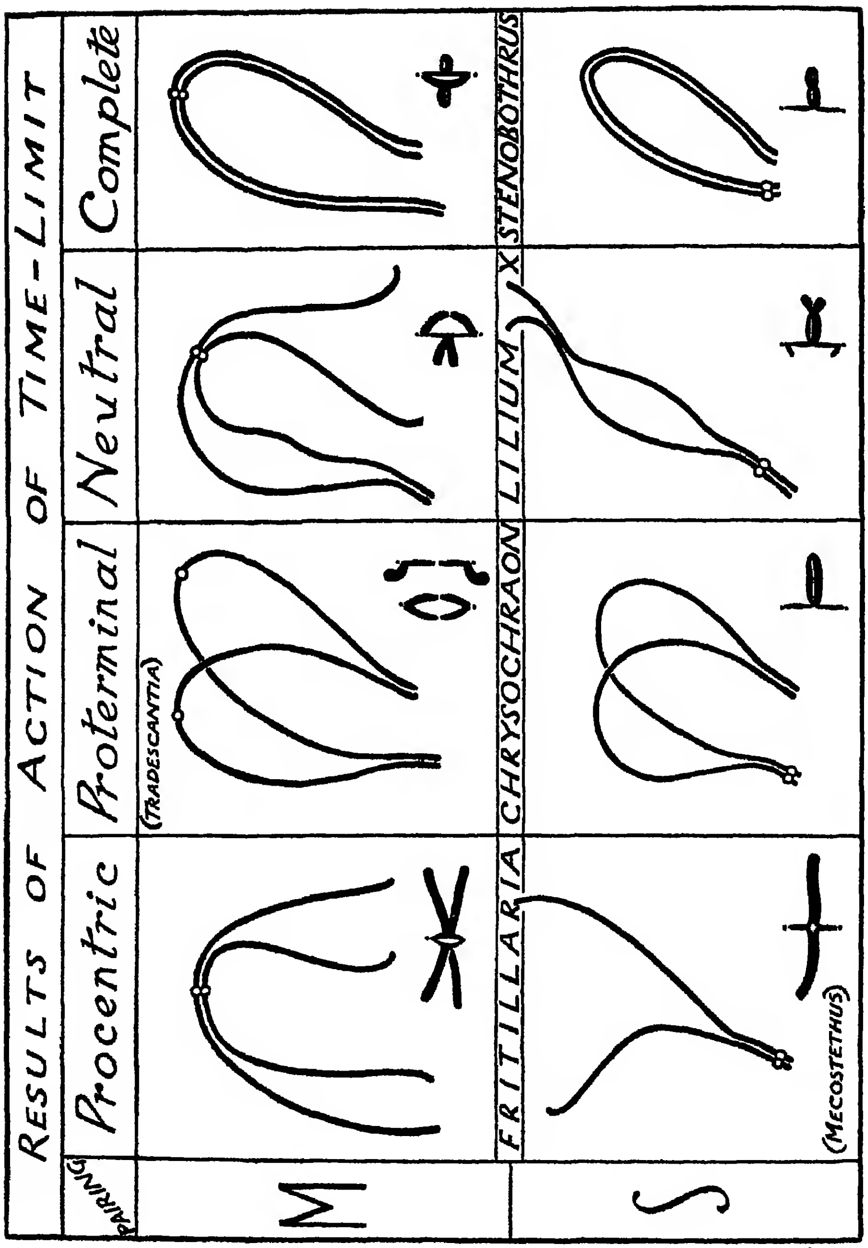


Fig. 1. Diagrams of pachytene and metaphase bivalents showing the comparative effects of procentric and proterminal pairing with time limit and of complete pairing without a time limit on chromosomes of *M* and *S* types.

only such parts will usually pair and cross-over as are enabled by their position to do so early, i.e. before the time limit takes effect.

The question now arises as to whether there can be any other kind of localization than a proximal one. If localization is due to interruption of pairing there should be two kinds of localization corresponding to the two first contact points or places of initiation of pairing, namely proximal or centric and terminal or non-centric, as in *Fritillaria* and *Stenobothrus* respectively. If we turn to the relatives of *Stenobothrus* we find in *Chrysochraon* the terminal localization that is to be expected with a terminal initiation of pairing. *M* and *S* chromosomes equally have two chiasmata, one at each end. In the *S* chromosomes of course one of these is next to the centromere, and the loop produced resembles that in *Mecostethus* with incomplete centric localization. The distinction between terminal and centric localization is not therefore to be determined, not absolutely from the structure of individual bivalents, but relatively from statistical comparison, the behaviour of *M* chromosomes being the test of priority or non-priority of the centromere in pairing. There is no evidence yet that the centromere can ever have a negative or deterrent effect on the initiation of pairing. Its action can be only positive or neutral. And when it is neutral the freedom of the ends gives them an inherent advantage. For this reason the antithesis between organisms in which the centromere is favoured and those in which the ends are favoured can be conveniently described as *procentric* against *proterminal* initiation or localization. It is possible that there is a third limit of behaviour where the start of pairing is free, neutral or indeterminate.

Proterminal localization is to be inferred in organisms with *M* chromosomes alone, like *Allium Cepa* and the tetraploid *Tradescantia virginiana*, as well as in organisms of mixed *M* and *S* types like *Chrysochraon*. In *Tradescantia* Pätau (1940) has shown, in accordance with this view of end-pairing, that the frequencies of association of from one to seven chromosomes to be expected in an aneuploid interchanged *Tradescantia* with 22 chromosomes can be predicted from the assortment of each end of each chromosome with a fixed, equal, and independent chance of pairing and chiasma formation with each of its two or three possible partners—this chance being the pairing coefficient, *w*.

We now have to turn from the position types of initiation of pairing to the modes of operation of the time limit. The systems of localization we have considered are all characteristic of species. They are due to the action of a time-limit genotypically enforced. It is the hereditary property of these species to have both a regular position of the contact points and a regular reduction in the time available for pairing to less than is necessary for its completion; to less indeed than is available in related species.

Can the time limit come into operation by other means than natural adaptation? We might suppose that any slowing down of pairing would have the same effect of cutting it short. Again the comparative method provides us with the means of testing our hypothesis. Mutants, hybrids and tetraploid derivatives can be compared with their immediate progenitors. But we may begin by considering the extremes of variation occurring under supposedly normal conditions in species with usually unlimited pairing.

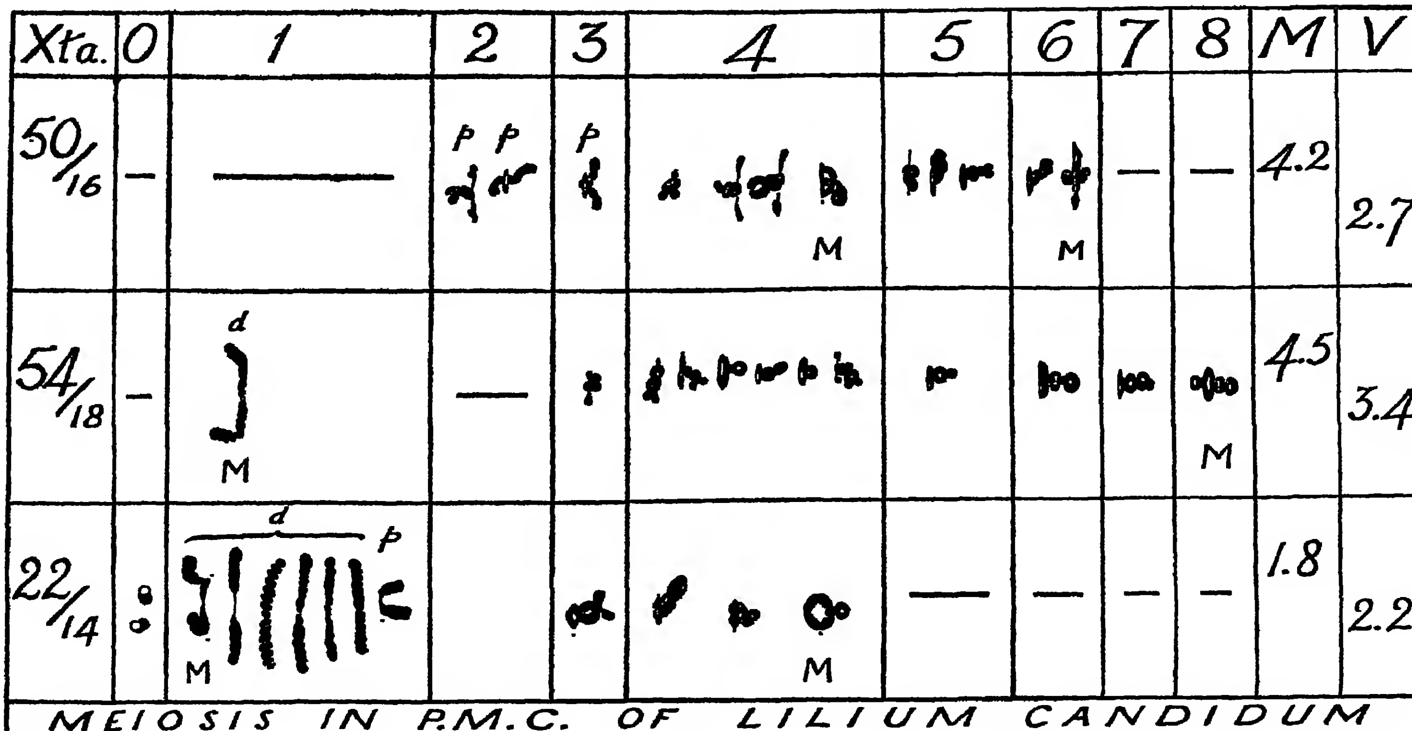


Fig. 2. Bivalents of three pollen mother cells of *Lilium candidum*, classified according to chiasma frequency. Above, pairing nearly complete and otherwise procentric; middle, nearly complete but one bivalent distally localized; below, unco-ordinated, largely incomplete and then evidently proterminal. (Unpublished observations of Dr K. Mather.)

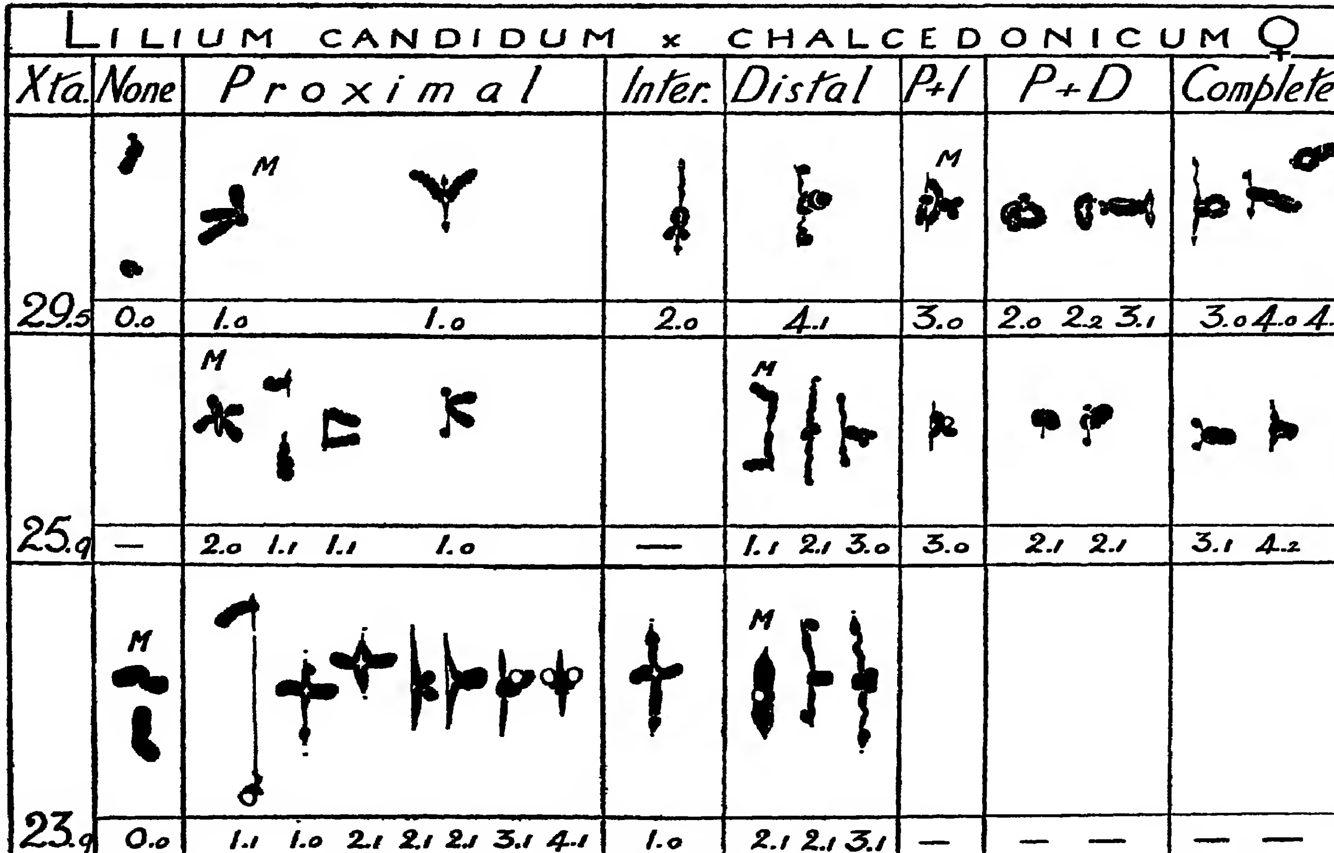


Fig. 3. Bivalents of three embryo-sac mother cells of *Lilium testaceum* classified according to the points of contact and resultant distribution of chiasmata. Numbers of total and terminal chiasmata under each bivalent. The nuclei have a similar chiasma frequency but different distribution and a general lack of co-ordination (Darlington & La Cour, 1940).

The first indication of such effects is to be seen in Mather's material of *Lilium candidum*. Particular cells may include, along with the normal bivalents having three to six chiasmata evenly distributed, others having single chiasmata near the centromere as in *Fritillaria*, and others again with single terminal chiasmata as in the tetraploid species of *Tradescantia* (Fig. 2). A similar exception has been illustrated in *Trillium ovatum* (Warmke, 1937). These sporadic cells indicate the action of the time limit in plants with slightly procentric pairing. And further their very rarity implies an accurate regulation in the time limit of the species concerned, a regulation which being upset in the exceptional cells leads to the interruption of pairing before it is complete.

But there are several other possible causes of time limitation. The most obvious is the slower or less perfect pairing that is to be expected in structural hybrids. Taking the species crosses of *Lilium*, in those nuclei with the 40 or so chiasmata usual in the parents there is the same even distribution of these chiasmata as in the parents. But nuclei having only 10 or 20 chiasmata (and having consequently several unpaired chromosomes) show a pronounced change of distribution. The chiasmata occur in groups near the centromeres or near the ends. The whole of the bivalents in a cell may be of the type found with extreme procentric localization in *Fritillaria*. Again the *M* chromosomes, which show an especially increased variance in chiasma frequency, suffer most from lack of pairing in these cells (Darlington & La Cour, 1940). The selection of cells with low chiasma frequency is thus a selection for early time limit and enables us to trace the stages of pairing in an individual whose average properties betray no peculiarities in the distribution of chiasmata.

The same method can be applied to triploids. Average cells of the triploid *Fritillaria latifolia* show little sign of the centric localization found in the diploid. The increased opportunities of pairing and consequently increased chiasma frequency of the triploid generally mask its localization. Only one of the three chromosomes of a trivalent ever shows centric localization. Statistically, however (Fig. 3), the *M*/*S* relationship reveals the procentric habit. *M*'s suffer more than *S*'s from the procentric localization, as in the diploid. They suffer also in another way, namely that they have on the average fewer separate points of contact or effective pairing blocks (1·75 as against 2·09). The same property is shown by a different kind of statistical analysis in triploid *Hyacinthus* (Stone & Mather, 1932).

In hybrids of species with none but *M* chromosomes the regular formation of only terminal chiasmata has long been recognized. The "loose pairing" of *Triticum-Aegilops* crosses is of this kind. Evidently in this group pairing begins at the ends. We should expect the same proterminal localization in mutants and segregates of related cereals with reduced pairihg. Lamm's inbred rye in fact shows lower spiralization together with the partial failure of metaphase pairing that is to be expected from reduced precocity. Like the wheat hybrids it shows proterminal localization increasing as the chiasma frequency is reduced. Considering *Triticum* from this point of view we can regard the rare occurrence in *T. vulgare* of a bivalent with five chiasmata as the converse of the occurrence in *Lilium candidum* of a bivalent with only one chiasma (Frankel, unpublished).

At this point it may well be asked whether the time limit can be imposed artificially. Mather (1934) found that a delayed effect of X-ray injury on *Vicia Faba* was localization of chiasmata as well as an increase in their frequency in those segments where they were formed. Barber (1940) by keeping *Uvularia* pollen mother cells in prophase at 30° C. for 2 days has cut short chromosome pairing and at the same time produced a procentric localization of such chiasmata as are

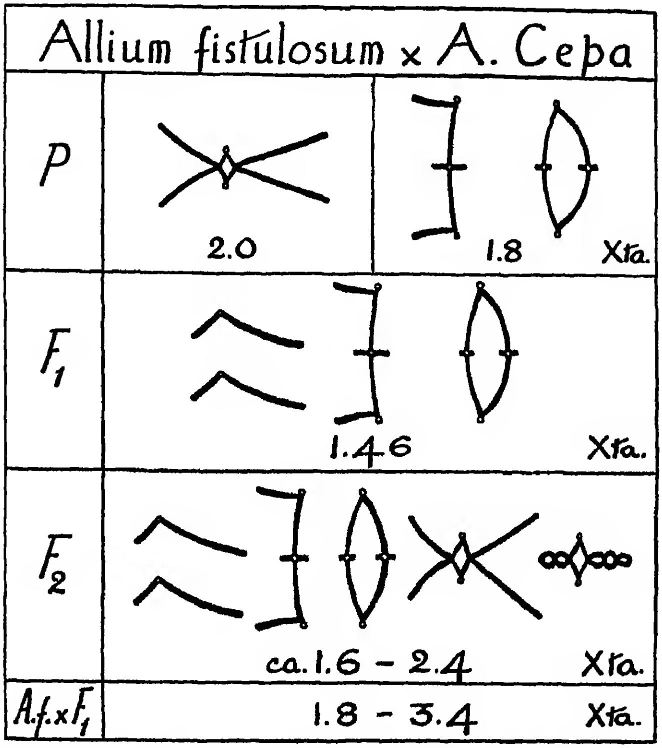


Fig. 4. Chiasma frequency and distribution in two species of *Allium*, in their cross and in its derivatives. Numbers are average frequencies for individuals. In the $F_2$ all types shown may occur in one individual. The occurrence of the last type proves that the two preceding types, occurring in the same nuclei, are due to localization and not movement. Recombinations in the $F_2$ prove that the three prime variables of meiosis are genetically independent in the $F_1$. There is also evidence of inversion and interchange hybridity in the $F_1$. (After Levan, 1936; Maeda, 1937. Reproduced from *The Evolution of Genetic Systems*, 1939, p. 69.)

formed. In this plant pairing is normally complete and has not been seen in progress, but the effects of its interruption show that it begins near the centromere. Again with procentric localization the *M* chromosomes suffer more than the shorter *S* chromosomes. The ordinary rules of proportionality of chiasmata to length, Haldane's interference and Mather's competition, equally break down when pairing is interrupted.

The genetic basis of these variations is revealed in a diagrammatic way by the results of a species cross, Maeda's (1937) observations on the second generation of an *Allium* hybrid (Fig. 4). The cross between one species with procentric, and another

with proterminal, localization gave an $F_1$ with proterminal localization. The $F_2$ was remarkable both in range and in individual character. To take first mere gross chiasma frequency: some plants as usual had a lower frequency than either parental species. But others had a higher chiasma frequency, an unprecedented consequence of segregation. Examining these plants we find that the reason lies, not in a general and evenly distributed increase, but in the removal of localization in some of the bivalents. Their pairing has evidently been completed so that chiasmata are formed throughout their length. Again we have the converse of the situation in the *Lilium* hybrids. What does this mean? Elements of genotypes determining a reduced time limit and different places of contact have been recombined. The recombination has in some plants extended the time limit, in others reduced it. And generally the uniformity and co-ordination of cells and individuals as to the point of contact and the time available for pairing are upset. One cell may contain bivalents with proximal and with distal localization and with no localization at all.

The mechanical implications of this breakdown are important. It might have been supposed that the terminal chiasmata of *Allium Cepa* were the result of movement. But when we see bivalents with such chiasmata side by side in one cell with others having proximal localization we must admit that movement is responsible for the positions of the chiasmata in neither case. The metaphase positions imply an original localization in species and crosses alike. The same conclusion is reached by Mather (1939) from a comparison of positions and frequencies of chiasmata in many organisms with large chromosomes.

Parenthetically it should be said that this segregation in the *Allium* hybrids shows a genetic attribute of species not otherwise so clearly demonstrable. The meiotic systems of the two parental forms were evidently buffered or balanced in two different ways to give two kinds of regular result. This accurate adjustment is upset by segregation in the $F_2$ to give plants with a method of pairing which although superficially regular is in detail seen to be unco-ordinated. Hitherto co-ordination in the points of contact had been so invariable as to appear inherent in the nature of the process. Occasional exceptions like the aberrant *Fritillaria pontica* described by Frankel and those *Oenothera* trisomics with interstitial chiasmata were not flagrant enough to demand an explanation. Now the evidence of *Allium* bears out the indications we have seen in the odd cells of *Lilium* and *Triticum* and bears them out overwhelmingly. Co-ordination it appears is one of the prime variables of meiosis in spite of being one of the prime invariables of species.

So far we have been able to assume that the action of a time limit stops pairing prematurely in all these examples of localization and restriction. This explanation is simple. It follows directly from the precocity theory and the causal sequence it implies. It has moreover been verified by observation of the active stages in diploid *Mecostethus* and *Fritillaria* as well as in structurally hybrid and tetrasomic *Zea mays*. The proportion of the lengths of chromosomes associated at pachytene is not however the only variable at work. We already know that chiasma frequency varies in different clones of *Fritillaria imperialis* all with nearly complete pairing. Torsion of the paired chromosomes, or whatever else is responsible for their

crossing-over potential, must therefore vary. Again, parts of chromosomes, and even whole chromosomes particularly, in "asynaptic" mutants of *Zea*, *Crepis* and *Allium* (Beadle, 1933; Richardson, 1935; Levan, 1940) can pair at pachytene and then fall apart without crossing-over.

It is easiest to understand these abnormalities on the assumption that the torsion developed within and between paired chromosomes varies along their length and as between different bivalents and different individuals. Torsion would then have a certain threshold value for crossing-over, below which it was ineffective, and the amount of crossing-over that was to take place, the *crossing-over potential*, would be proportionate to the amount of torsion above this threshold.

We must now consider the means that have been used to test these possibilities. Other possibilities, such as the conditioning of the threshold, can be set aside for the moment.

If differences in torsion occur in comparable pairs of chromosomes at pachytene they must be related to the same timing differences as the all-or-nothing differences in pairing, and for the following reason. Unpaired parts of chromosomes can develop relational coiling during pachytene. This coiling, when it has taken place between two contacts, can still be seen at diplotene in *Fritillaria Elwesii*. Chromosomes can therefore undo their internal twist before they are paired. And it seems they are bound to do so likewise if they pair late (although to a less degree). In short if they pair late they will pair with their torsion partly lost. Further, the association of two chromosomes is not instantaneous, so that different parts will have different torsions (cf. Darlington, 1935*b*, Fig. 29). The amount of coiling surviving at diplotene in asynaptic plants should then correspond to a pachytene coiling insufficient to have reached the crossing-over threshold.

There is another and more roundabout way of testing the effects of differential torsion. The length proportions of *M* and *S* chromosomes are the same within narrow limits throughout *Lilium* and *Fritillaria*—about 10 to 7. In *Lilium* species with complete pairing the chiasmata are formed in this proportion. But where the pairing is strongly procentric and is interrupted as in many *Fritillaria* species, the proportion changes to equality. As we noted, *M*'s suffer more than *S*'s from a reduction of pairing. If however there is differential torsion even in *Fritillaria* species with complete pairing, the unfavourable situation of the *M* chromosomes should express itself, although less strongly, in these species. Frankel's observations leave no doubt that this is so (Fig. 5).

The same disadvantage overtakes the *M* chromosomes in *Lilium* hybrids, which are less procentric than *Fritillaria* species. Here pairing is interrupted before it is complete and the earlier it is interrupted the lower the relative frequency of the chiasmata in *M* chromosomes. It seems that the obstacle effects of hybridity fall more heavily on the larger chromosomes irrespective of the genotypically determined point of contact. They demand new points of contact and new movements of intercalary parts. In these movements the greater size means the greater delay, and the greater delay means the greater chance of the pairing being too late. In a different way the same end is reached.

In thinking of the effects of torsion we have to remember the crossing-over properties of triploids. In triploids chromosomes pair two-by-two and each chromosome on an average can pair only for two-thirds of its length. Nevertheless the aggregate amount of crossing-over per chromosome is generally about the same in triploids and diploids. Its distribution however is different, and this provides the

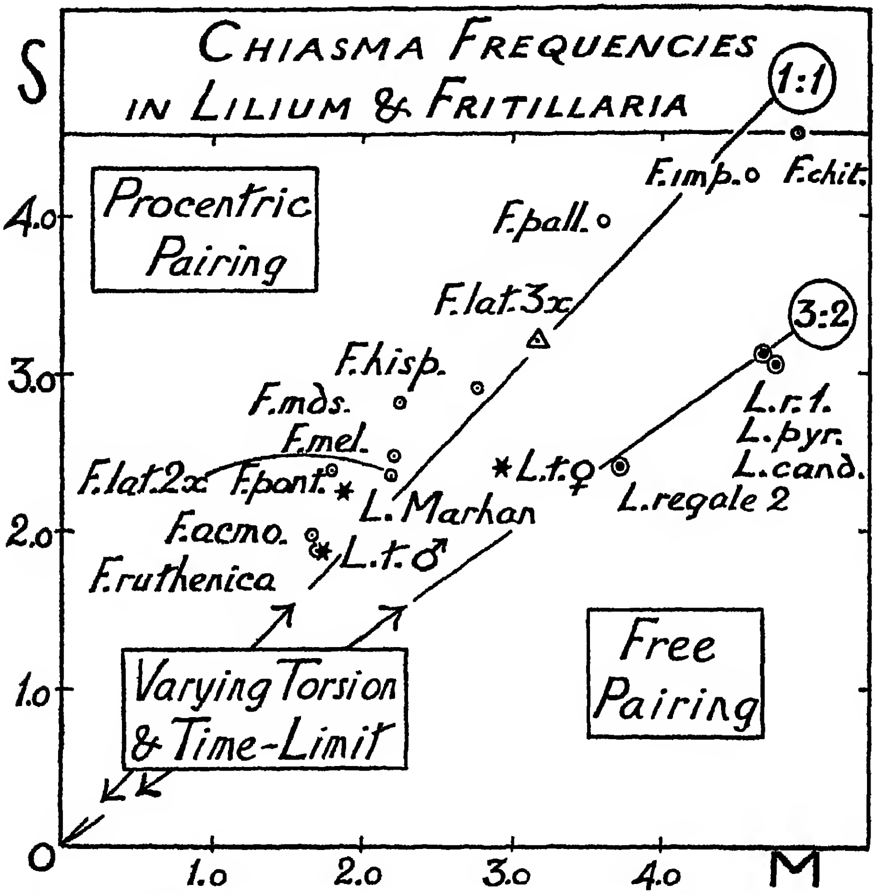


Fig. 5. Graph showing relative chiasma frequencies of *M* and *S* chromosomes. 3 : 2 line shows approximate proportionality to length. Diploid *Fritillaria* species from Frankel (1940). Triploid *Fritillaria* from Darlington (1940). *Lilium* species from Mather (unpublished). *L. Marhan* from Richardson (unpublished). *L. testaceum* from Darlington & La Cour (1940).

solution of the problem. For, in triploids, the chromosomes make contact at more points independently than in diploids or tetraploids (Upcott, 1939*a*). They therefore anchor one another and prevent that uncoiling which, with only one point of contact, will allow a wastage of crossing-over potential. This conclusion is borne out by the disproportionate increase of chiasma frequency found in configurations with one change of partner, and therefore two points of contact, over simple bivalents in triploid *Tulipa* and in the *Fritillaria latifolia* already referred to.

This is true of the *S* type of chromosomes where the two anchorage points are usually at opposite ends of the chromosomes. But in *M* chromosomes the two points are close together, on either side of the centromere. The anchorage can have no effect in preventing uncoiling. We then find that in these chromosomes the change of partner makes no difference to the chiasma frequency.

Other evidence leads to the conclusion that differential torsion within each pair is characteristic of all chromosome pairing and is responsible for a greater determinacy in the position and frequency of crossing-over than is at first apparent. The combined statistical and individual observations of chiasma frequency and distribution that Mather (1939) has brought together in discussing ***competition*** and ***position-determination*** indicate that the torsion developed in the paired chromosomes is not uniform throughout their length. It has a characteristic relationship with the centromere, or perhaps with the place where pairing began, and also characteristic properties for the nucleus as a whole.

For these deductions from metaphase chiasmata we have to confine ourselves to large chromosomes where terminalization has caused little disturbance of position. But now these rules can be applied to the mere frequency of chiasmata and crossing-over in plants and animals generally. If the same time is available for pairing in a new tetraploid as was just sufficient for completeness in its parents, less pairing or less early pairing will take place and fewer chiasmata will be formed. Upcott's comparison (1939*b*) of chiasma frequencies in diploids and tetraploids shows how this principle can be used. The ***reduction factor*** reaches its highest effect (0·84) in chromosomes of the largest size and is extremely slight (0·97) with the smallest chromosomes. There is one clear exception to the regularity of this order, the orthopteran *Schistocerca*, where we know that the pairing is helped by a special orientation and contraction during the active stages. The reduction factor is nearer to unity than in comparable plants (Table I).

These various kinds of evidence together establish certain new principles and at the same time reveal certain new problems. The principles concern the prime variables of meiosis. These are three:

First, the ***contact point***, the position where pairing begins; whether ***procentric***, at the centromere without regard to the ends, or ***proterminal***, at the ends without regard to the centromere, or intermediate between these two extremes and therefore mixed in result.

Secondly, the ***time limit***, or time available for pairing, whether enough to allow of its completion or so short as to lead to its interruption and consequent localization near the contact point.

Thirdly, the amount of ***torsion*** capable of being developed in different parts of the chromosomes after pairing, whether enough to reach the threshold for crossing-over or so little as to allow the chromosomes, wholly or in part, to separate without crossing-over when they divide.

To these a fourth may be added of a physiologically different character: the presence or absence of co-ordination amongst the chromosomes and cells of individuals in regard to the first three variables. This co-ordination is characteristic of stable species because it is in fact necessary for their stability.

Table I. *Causes of crossing-over restriction*

| Interruption of pairing by action of time limit | Start of pairing: Procentric | Proterminal | Intermediate or mixed |
|---|---|---|---|
| Through early time limit: | | | |
| (*a*) Species | *Mecostethus*<br>*Fritillaria*<br>*Allium fist.* | *Bombyx* ♀<br>*Chrysochraon*<br>*Allium Cepa* | *Paeonia* |
| (*b*) Mutants and segregates | — | *Zea*<br>*Pisum*<br>*Secale* | *Crepis X*<br>*Allium $F_2$** |
| (*c*) Treated or conditioned | *Uvularia* | *Lilium candidum* | — |
| Through delay in pairing: | | | |
| (*a*) Tetraploids | — | *Tradescantia* | Reduction factor types |
| (*b*) Structural hybrids | *Lilium* hybrids* (*S* type) | *Triticum*<br>*Paeonia* | *Lilium* hybrids* (*M* type) |
| (*c*) Haploids | — | *Triticum*<br>*Oryza*<br>*Nicotiana* | — |

* Partially unco-ordinated.

The problems that arise concern the genetic and mechanical control of the point where pairing begins; the influence of the shapes and positions of chromosomes at telophase and early prophase on this point of first contact; the relative importance of non-pairing and non-torsion in the restriction of crossing-over; the effect of triploidy on localization; the different effects of different kinds of experimental interruption of pairing; the relation of localized crossing-over to the localized activity of genes. These and similar problems can now be approached.

Meanwhile, those simple changes that are inherent in our basic type of meiosis are now becoming clear. Their study is undoubtedly necessary for our ultimate understanding of the causal sequence of meiosis, and it seems to be of immediate use in showing what limits must be set to the use of meiosis as the mechanism of genetic recombination.

SUMMARY

1. The uniform series of chromosome movements we know as meiosis (pairing, torsion, reproduction, crossing-over, co-orientation, segregation) can now be placed in a causal sequence. This serves two purposes: to be tested as a working hypothesis and to be applied to the understanding of the characteristic variations of which meiosis is susceptible.

2. Comparison of mutants, hybrids and other genetically controlled variants shows that these variations arise from three main sources: (i) the point at which the pairing chromosomes make contact; (ii) the time available for pairing; (iii) the amount of torsion capable of being developed in the parts of the chromosomes which are paired.

3. All species are, as such, characteristically co-ordinated with regard to these variables. They may be classified as procentric (*Mecostethus*) or proterminal (*Chrysochraon*) in the initiation of pairing. This difference affects the relative frequency of crossing-over in chromosomes of two main types, with the centromere near an end and away from it (*Fritillaria, Lilium*).

4. Exceptional cells, and individuals arising by segregation from hybrids, show that co-ordination within the cell and the individual is not physiologically inherent in meiosis. Unco-ordinated behaviour can take place in regard to all three prime variables (*Allium, Lilium* and *Trillium*).

5. The time limit to pairing may be imposed artificially by heat treatment or X-raying. We then have artificial localization of crossing-over (*Uvularia, Vicia*).

6. The amount of torsion depends on the speed of pairing and this in turn on the size of the nucleus. Doubling the chromosome number therefore reduces the crossing-over frequency (*Primula, Allium, Solanum*, etc.).

7. The preservation of torsion depends on the early contact of a chromosome at two points. The more numerous contact points of triploids therefore increase their frequency of crossing-over per unit length paired, at the same time changing its distribution (*Fritillaria, Tulipa, Drosophila*).

8. All species hybrids are structural hybrids. Their pairing is therefore slower and is cut short by the time limit. Hence their crossing-over is reduced and relatively localized, unless the extreme of localization has already been reached in the parents (*Triticum, Lilium*).

9. The study of the three prime variables is therefore necessary for the understanding of the causal sequence of meiosis and of the conditions of stability and coherence in species.

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# FLUORESCENCE MICROSCOPY IN BIOLOGY

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(*Received* 1 *January* 1940)

CONTENTS



## I. HISTORY OF FLUORESCENCE MICROSCOPY

OWING primarily to the researches of Abbe, microscopy in transmitted light reached a high degree of perfection at the close of the nineteenth century. Three optical factors determine the resolving power of the microscope, viz. (*a*) the magnification, (*b*) the aperture of the cone of rays taken up by the objective, and (*c*) the geometric accuracy of the junction of the rays at the point of the image. As Abbe (1878) showed in 1878, the theoretically possible optimum conditions were already almost reached as regards factors (*a*) and (*b*), while the third (*c*) was being realized with the invention of apochromatic lenses (Abbe, 1886). Since the resolving power could not be increased by improving the optical conditions, other means had to be found to achieve this. The reproducibility of an object reaches its lower limit when the dimensions of the features to be reproduced are about ten times the wave-length of the light used (Abbe, 1873). By the use of ultra-violet instead of white light the limit can be reduced to one-half. Starting from these considerations Köhler (1904) constructed the first ultra-violet microscope. Considerable difficulties in the use of this microscope when observing biological materials were due to frequent fluorescence of such subjects when exposed to ultra-violet light. Köhler (1904), however, recognized immediately that it would be possible to make use of the fluorescence of such subjects for the purpose of microscopy. According to Helmholtz (1874) it was

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to be expected that the image would be better differentiated and the fine structures more easily discernible if the microscopic object itself were to emit light. It is not certain that it will be possible to achieve an increase in magnification using fluorescence microscopy, which Baum (1932) claims should be obtainable, owing to the absence of refraction phenomena with this method. On the other hand, the contrast between the light-fluorescent and the dark non-fluorescent patches becomes more marked with this method than with the usual microscopy in transmitted white light. In addition, fluorescence microscopy permits one to observe the presence of spontaneously fluorescent substances in the tissues and with it the chemical differentiation of the biological structure such as cannot be seen in the stained section. The light of the line of 275 mμ of the cadmium spark was used for illumination in an arrangement constructed *ad hoc* by Köhler & Siedentopf (1908) for demonstration at a vacation course for scientific microscopy held in Vienna in 1908, and this may be considered to be the first attempt at a fluorescence microscope. Satisfactory work, however, became possible only when Lehmann (1910) succeeded in using the far more intense light of the carbon arc as a source of ultra-violet light by introducing suitable filters. He combined a nitrosodimethylaniline filter of Wood (1903) which absorbs the yellow, green and orange, with a blue Uviol glass filter to absorb blue and violet, and with a copper sulphate filter to absorb the red and infra-red. This arrangement allowed only rays of the wave-length 280–400 mμ to pass. Almost simultaneously the firms of Carl Zeiss and C. Reichert constructed the first ultra-violet microscopes (Lehmann, 1913; Reichert, 1911; Heimstädt, 1911) on this principle. The main difference between these lay in the illumination. That of Zeiss used light-ground and that of Reichert dark-ground condensers. Both could be used only with transmitted light, and both employed quartz lenses in the illuminating apparatus and normal glass lenses in the microscope. They corresponded in their construction almost entirely to the most modern models which will be described later. Few publications concerned with fluorescence microscopical researches date from these early days. While Stübel (1911) examined the spontaneous fluorescence in animal objects and Klemm (1917) that occurring in plants, v. Prowazek (1914) tried to stain non-fluorescent objects with fluorescent materials, thus making these open to observation by fluorescence microscopy. Attempts to use the method for the purpose of recognizing and differentiating bacteria were made comparatively early (Kaiserling, 1917, 1921; Arloing *et al.* 1925). There was a considerable advance when Derrien (1924) succeeded in localizing porphyrins in different animal tissues. The method received a fresh impetus when Haitinger in collaboration with Hamperl (Haitinger & Hamperl, 1933) and Linsbauer (Haitinger & Linsbauer, 1933, 1935; Haitinger, 1935) elaborated systematically a technique for staining animal and plant objects with fluorescent substances which he called fluorochroms, and when the firm of C. Reichert, Vienna, placed on the instigation of Haitinger, fluorescence microscopes at the disposal of numerous laboratories. The introduction of the method into virus research by Hagemann (1937 *a, c*) possibly opens up a new field.

From an entirely different point of view Ellinger & Hirt (1929 *a, b*, 1930 *b*)

developed a fluorescence microscope for incident light and used it for the observation of processes in the living organism, a method called by them "Intravital microscopy". Microscopy of the living organism was fairly rudimentary at the time. For examination of living organs it became necessary, owing to the size and opacity of the object, to use incident illumination. Depending on the subject used much of the incident light was either back-reflected from the surface or absorbed by the superficial layers of the object, being thus at any rate lost to the formation of the microscopic image. The proposal of Vonwiller (1927) to employ the absorbed light in the formation of the image by introducing artificial reflectors into the tissues, proved itself to be a method of little use. For effective progress here it became necessary to place a source of light in the object itself and further to exclude interference of the light back-reflected from the surface of the object. This would be achieved only when the light of the primary light source and that excited in the object and used for the illumination of the microscopic image, differed in wavelength to such an extent that they could be separated entirely by suitable filters. This could be attained by using ultra-violet light for illumination, by treating the living object with fluorescent substances, and by interposing filters impermeable to the exciting rays but permeable to the fluorescent light between objective and eyepiece. An intravital microscope built by Zeiss according to the specification of Ellinger & Hirt (1929*b*, 1930*b*) was followed by a number of similar constructions by other firms [E. Leitz; Bausch and Lomb instructed by Singer (1932); C. Reichert instructed by Pick (Mehler & Pick, 1932; Pick, 1934), which, however, neglected a number of important features of the original method]. In special cases, as for instance in the microscopic observation of the living eye with the corneal microscope, ultra-violet light of the slit lamp had already been used after injection of fluorescin by Thiel (1926). Details of intravital microscopy and of the results obtained by it will be discussed later. Though investigations by this method have been conducted only in comparatively few places a considerable number of problems of animal and plant physiology have been solved with it, indeed, a new group of natural pigments which includes a constituent of the vitamin $B_2$ complex has been discovered and isolated by this method (Ellinger & Koschara, 1933 *a*, *b*, *c*, 1934). Recently, further progress has been made, so that by increase of the speed of the photographic emulsion it has become possible to reproduce the image obtained photographically and even by colour photography.

## II. TECHNIQUE

### (1) The apparatus

Like all microscopes the fluorescence microscope consists of three different parts, the source of light, the illuminating apparatus, and the actual microscope.

#### (*a*) *The source of light*

The essence of fluorescence microscopy is the use of the light source to excite the fluorescence of fluorescent substances either already present in the object or injected into it previously, so that the fluorescent light overtakes the illumination of the

object while the light of the primary source of light is removed by suitable filters after having served its purpose so as not to interfere with the clarity of the image. Fluorescence is the property of a substance which effects the conversion of a part of the absorbed light energy into light of a wave-length different from that of the exciting rays and characteristic for the fluorescent material. According to Stokes's law this fluorescent light must always be poorer in energy, i.e. of longer wave-length than the exciting light. It is thus essential to choose a short wave-length for the exciting light, in order to cause the fluorescence to cover the whole spectrum if necessary. Thus ultra-violet light has come into common use as the source of light. The use of invisible light for the exciting radiation has the advantage that the visual observation of the fluorescent image is not disturbed by visible reflexes from the surface of the object. Continuous exposure of the eye to ultra-violet light, however, is inadvisable. If one deals with fluorescent light of longer wave-length (green to red) there is no objection to the use of visible light of short wave-length in addition to ultra-violet, so long as it is possible to keep this from the eye or camera by the use of suitable trap filters, and so long as it does not interfere with the quality of the fluorescent image, e.g. when acriflavin is used in intravital microscopy, the exciting light may contain the violet and blue in addition to the ultra-violet.

Thus a lamp the light of which is rich in ultra-violet rays is chosen for illumination. The lamp must have a sufficient intrinsic brightness, burn regularly and must be easy to handle. The 275 m$\mu$ line of the cadmium spark used in the first fluorescence microscope (Köhler & Siedentopf, 1908), being a source of light of far too low intensity, was at once abandoned. The fluorescence microscope by Reichert-Heimstädt (Reichert, 1911; Heimstädt, 1911) used an iron carbon arc lamp, that by Lehmann (1913) an iron or nickel (cf. also Metzner, 1928) carbon arc lamp, the electrodes of the latter being arranged at right angles so as to attain a better field of light. Later, different authors have recommended the most divergent sources of light, e.g. the normal type of mercury-vapour lamp (Metzner, 1930 *a*; Haitinger, 1930; Vodrazka, 1930 *a*) including one of annular shape and arranged round the objective (Daugherty & Hjort, 1934), the intensity of which is entirely inadequate; the high-intensity mercury lamp (Barnard & Welch, 1937; Gerlach, 1937 *a*; Melczer & Venkei-Wlassics, 1939); a tungsten arc *in vacuo* (Kögel, 1928); sparks between magnesium (Barnard & Welch, 1936) or iron (Singer, 1932) electrodes; the slit lamp (Thiel, 1926; Henning, 1932 *a*); and an arc between carbons with iron wick (Haitinger, 1931, 1934, 1938 *a*, *b*); the use of a paraboloid mirror was recommended to increase the light intensity (Kögel, 1928). The most suitable arrangement seems to be the carbon arc lamp with carbons at right angles for 10 amp. D.C., which can be overloaded for a short time to 20 amp., adjusted by clockwork (Zeiss, 1939). The iron wick carbon lamp (Haitinger, 1938 *a*, *b*) gives also satisfactory results with 5 amp. D.C. The most recent models of high-pressure mercury-vapour lamps are still far inferior in intrinsic brightness to the most effective carbon arc lamps (Melczer & Venkei-Wlassics, 1939). If, however, the former of the same intrinsic brightness as the latter can be constructed, these would be preferable, since they are handled more easily and do away with unpleasant combustion gases.

The light of such light sources is then suitably filtered to remove the infra-red and a more or less extensive portion of the visible rays.

In most of the more recent models Uvet glass filters of 0·5–5 mm. thickness which are impermeable for the visible light apart from the extreme red replace the combination of the nitrosodimethylaniline and blue Uviol glass filters. These filters are generally placed in the light beam behind the cuvette containing copper sulphate (4–25 % layers, of different thickness) removing red and infra-red rays. It is still difficult to find the correct filters for the examination of substances emitting pink or red fluorescence (i.e. porphyrins) as Uvet glass and copper sulphate filters, sufficiently thick to keep back the last traces of the red rays of the light source, absorb a considerable proportion of the ultra-violet and thus weaken the exciting light. In some models the thickness of the Uvet filters can be varied. In the intravital microscope Uvet filters can be replaced by blue Uviol glass filters of 1–5 mm. thickness permitting violet and blue light to pass in addition to ultra-violet if required. The light of the high-pressure mercury-vapour lamp contains so little red that copper sulphate filters are wanted only in special circumstances (Melczer & Venkei-Wlassics, 1939).

### (*b*) *The illuminating apparatus*

The two types of fluorescence microscopes, viz. those designed for transmitted and for incident light, respectively, require different types of illuminating apparatus. The former can be used only for translucent objects such as tissue sections, smears, bacterial cultures or tissue cultures, while the latter can be used for such objects as well as for opaque ones, e.g. for the observation of living organs in situ.

The first part of the illuminating apparatus is the same for both types. Quartz lenses (back lenses of a quartz condenser) render the beam of the light source parallel. Red light is removed and the beam is cooled by water and copper sulphate in plane parallel cuvettes with windows of quartz or of very thin glass permeable to ultra-violet light. Another quartz lens acting as the front lens of the quartz condenser, collects the rays and projects the image of the light source on to a totally reflecting quartz prism. The Uvet filter is usually placed in this part of the light beam. The rest of the apparatus differs in the two types.

In the light-ground fluorescence microscope for transmitted light (Lehmann, 1913; modern luminescence microscope by Zeiss (1939); Loos (1939); Haitinger (1931, 1938 *a*, *b*); fluorescence microscope of Bausch and Lomb according to Singer (1932)) the ultra-violet rays are deflected through a right angle by a quartz prism replacing the mirror, into an Abbe illuminator, with lenses of quartz or Uviol glass. Prism and illuminator are fixed in the substage. The Abbe condenser converges the rays on to the object. In the light path are iris diaphragms acting as illuminated field and aperture diaphragms, respectively. In the early models (Köhler & Siedentopf, 1908; Heimstädt, 1911) a quartz dark-ground condenser was used instead of the Abbe condenser. Köhler & Siedentopf (1908) expected that the entrance of ultra-violet light into the object would thus be prevented; this did not happen owing to the deflexion of light at the object. Because of the small aperture

of the dark-ground condensers this method has almost become obsolete. It has, however, been recommended lately by Barnard & Welch (1936) and Barnard (1937), who used with very good results a dark-ground condenser by Smiles (1933).

The first fluorescence microscope for incident light was developed by Zeiss according to specifications by Ellinger & Hirt (1929 *a*, *b*, 1930 *b*), especially for the observation of living organs at high microscopical magnifications. In this model the beam converged by the quartz condenser enters the tube of the microscope through a side arm and is deflected from a prism on to an optically flat and parallel plate and from this into the objective. Through the objective which thus serves as a light-ground condenser the rays are collected on the object. Prism and plate are arranged so that loss in aperture of the objective is reduced to a minimum in contrast to the prism and plate arrangements usually used for vertical illumination (cf. Singer, 1932). The side arm bears aperture and illuminating field diaphragms whose use is essential in order to avoid overlighting of the object.

Apart from this inside illumination, outside illumination can be used for opaque objects. For objectives with a large object distance unilaterally incident light can be used, e.g. the filtered light of the slit lamp together with the corneal microscope (Thiel, 1926; Henning, 1932 *a*, *b*), or the light of a carbon arc lamp focused on to the object either by a paraboloid mirror (Metzner, 1929 *a*, *b*) or by a system of lenses (Schräglichtilluminator by F. Hauser, 1929) in connexion with a stereoscopic binocular dissecting microscope (Ellinger & Hirt, 1929 *a*; cf. also Turchini, 1924). The use of a "Schusterkugel" as condenser was recommended (Haitinger, 1930), but it appears to have been abandoned.

Circular outside illumination is used in the present fluorescence microscopes of Zeiss (Epicondensor W by Hauser, 1931, 1932, 1936; cf. also Schochardt, 1936), Leitz (Ultropak by Heine, 1931) and Reichert (Epilumilluminator by Ramsthaler, 1934 *a*, *b*). The ultra-violet light collected by the quartz condenser enters the microscope through a short side arm of the tube and is deflected through 90° into a paraboloid or cardioid condenser by an annular mirror centrally bored and inclined at 45° to the axis of the microscope and thus concentrated on the object. This type of illumination seems to have been first devised by Chapman & Alldridge (1923), but seems not to have been used before the Epicondensor W and the Ultropak had been constructed. These condensers act usually as dark-ground condensers. In addition to these condensers the original Lieberkühn mirror from which these condensers have been developed finds application in the observation of very small objects in the dark-ground microscope (Kögel, 1928; Hauser, 1931, 1932, 1936).

### (*c*) *The microscope*

Numerous authors claim that any normal microscope can be used for fluorescence microscopy in transmitted light, since the fluorescence is in the visible. The exciting ultra-violet rays, however, have to be removed by a cover-slip impassable to ultra-violet light. Heimstädt (1911), using a dark-ground condenser, does not even regard such a filtering cover-slip as essential. The protection against ultra-violet provided by such a cover-slip is actually insufficient, and fluorescent material

such as fluorite or plaster of Paris must be avoided in the objective. In order to exclude ultra-violet light back-reflected even from the surfaces of the objective lenses, lens mirror objectives were devised as substitutes for the usual microscope object glasses (Johnson, 1934). It is recommended to place an effective ultra-violet trap filter between objective and eyepiece so that there is at any rate no limitation to the use of the eyepiece. It is best to use slides of quartz, but some varieties of glasses are sufficiently permeable to ultra-violet light; it is essential only that the slides should not show fluorescence of their own. The same holds good for cover-slips, mounting materials and immersion fluids.

The use of filters which retain the exciting primary radiation entirely is essential both in fluorescent microscopy in incident and transmitted light. This has been recognized already by Lehmann (1913), who recommends Euphos cover-slips for the purpose. Later, a wide variety of filters for the purpose has been described. The arrangement of these filters on a sliding plate on the microscope tube between objective and eyepiece which permits easy exchange is recommended (Ellinger & Hirt, 1929 *a*, *b*, 1930 *b*). From the large number of excellent filters now available (Schott and Gen., Kodak, Ilford) one may choose with ease the most suitable type for any particular case. The spectral range must be adapted to the wave-length of the exciting rays and to the colour of the fluorescent light. It is essential that the thickness of the trap filter should be sufficient to cut out the primary radiation entirely, especially for photomicrographic purposes.

For fluorescence microscopy in incident light special microscopes are necessary, since light source and illuminating apparatus have to remain in a fixed position relative to each other. Therefore only stands with vertically movable stages can be used. The rough adjustment is done entirely by lifting and lowering of the stage, while the fine adjustment is made with the aid of the usual micrometer screw. In the arrangement of Ellinger & Hirt (1929 *a*, *b*, 1930 *b*) the objectives are made of glass readily permeable to long-wave ultra-violet light, since they are used simultaneously as condensers for the exciting rays. It is essential that the objectives show no fluorescence of their own, since in the microscopes with inside illumination the ultra-violet light passes the objectives before reaching the object. Conditions, however, are similar in the use of outside condensers, since a very large portion of the ultra-violet rays is back-reflected from the surface of the object into the objective not protected by filters. Franke's (1935) claim that with the Ultropak no ultra-violet light which might cause fluorescence of the objective passes into the microscope is incorrect. This can easily be demonstrated with the aid of photography. If Franke fails to obtain distinct images when using a vertical illuminator the cause is most likely to be found in the use of an inadequate microscope with fluorescent objectives. The use of microscopes built for other purposes in intravital microscopy appears to explain the unsatisfactory results of numerous researches. For the observation of living organs it is essential that water immersions without cover-slips should be used as objectives which are continuously irrigated by physiological salt solution in order to keep the surface of the object wet and to remove serous or coloured exudates. For investigations on warm-blooded animals the solution is

heated to 37° C., and a heated stage is used so as to keep the air surrounding the nimal at about 40° C. Small metal plates are attached to the stage permitting the fixation of the organ to be investigated (Ellinger & Hirt, 1929 *a*, *b*, 1930 *b*).

### (2) THE PREPARATION OF THE OBJECT

#### (*a*) *The preparation of objects for fluorescence microscopy in transmitted light*

For fluorescence microscopy in transmitted light special care has to be devoted to the preparation of the object. As fixative formalin is most suitable. For the observation of spontaneously fluorescent objects extraction of the fluorescent substances during the process of preparation has to be avoided. It is advisable to use frozen sections which are mounted in media permeable to ultra-violet light and not fluorescent themselves, e.g. water, physiological salt solution, glycerol, etc. (Heimstädt, 1911; Haitinger, 1938 *a*, *b*).

Objects not spontaneously fluorescent themselves may be prepared for observation by treating them with fluorescent substances as originated by v. Prowazek (1914). The method has been extended recently to plant (Haitinger & Linsbauer, 1933, 1935; Haitinger, 1935; Döring, 1935) and to animal material (Haitinger & Hamperl, 1933; Hamperl, 1934; Haitinger & Exner, 1936). For details the numerous publications should be consulted. Here, it must be kept in mind that there is frequently interaction between the tissue and fluorescent substance which may affect the fluorescence profoundly (cf. also Metzner, 1924). Thus fluorescence can be abolished by the heavy metal and nitrate ions when present in the tissue, or the fluorescence of the fluorochroms can be modified [*p*H dependence of the fluorescin fluorescence (Ellinger & Hirt, 1930 *b*; Déribéré, 1937)].

#### (*b*) *The preparation of living objects for intravital microscopy*

For intravital microscopy the objects are prepared as follows, according to the prescriptions given by Ellinger & Hirt (1929 *a*, *b*, 1930 *b*): the animals are anaesthetized (urethane, chloral hydrate) fixed to the stage, and the organ to be investigated is exposed and suitably fixed by means of the metal plate attached to the stage, and one must avoid upsetting the blood supply to the organ. The nature of this fixation must depend on the particular case. If spontaneous fluorescence is to be investigated, no further preparation is required. The organ is brought to the objective by lifting the stage which can be moved in three directions, and the region to be investigated is adjusted in white or blue light. Since the fluorescence of some substances is destroyed by prolonged irradiation with ultra-violet and even with white light, unnecessary exposure to light has to be avoided. Reversible and irreversible changes in staining and function due to prolonged irradiation have been reported (Ellinger & Hirt, 1931; v. Querner, 1932, 1935; Singer, 1936 *b*; Hirt, 1939 *b*). The actual observation of the object is carried out with continuous adjustment of the micrometer screw so that different levels are illuminated and focused, forming optical series sections of the object. Thus it is possible to investigate the superficial layers of a depth dependent on the free working distance of the objective and often considerable.

For the study of deeper layers the organ has to be prepared by taking shavings from the surface in a preliminary operation (Ellinger, 1934 *c*, 1935, 1938, 1940).

If the function of an organ is to be investigated injection of a fluorescent substance is necessary. This substance must not be poisonous and must fluoresce strongly in very low concentration. It must be chosen so as to stain if possible the cells of the organ to be investigated, though fluorescent material circulating in the blood stream suffice for the illumination of the picture. The most suitable dyes for intravital microscopy are fluorescin and acriflavin (Ellinger & Hirt, 1929 *a*, *b*, 1930 *b*). The former is particularly used for the study of the function of an organ; it stains predominantly protoplasm and body fluids, the latter cell structures. Fluorescin is also an indicator for the *p*H in cells and humours because the tint of its fluorescent light depends on the *p*H of the medium (Ellinger & Hirt, 1930 *b*). Other fluorescent vital stains as aesculin (Singer, 1932) or auramin and primulin (Hagemann, 1937 *a*, *c*, 1938; Keller, 1938) and others tested by Pick (1934 *b*) behave fundamentally like fluorescin or acriflavin, though they fluoresce less intensely and thus are not so effective; Rhodamin BS (Ellinger & Lambrechts, 1937 *b*; Strugger, 1938 *a*), which is intermediary between fluorescin and acriflavin, may become useful in special cases.

### (3) Photomicrography with the fluorescence microscope

Photography of inanimate objects in fluorescence microscopy presents no particular difficulties in spite of the comparatively low intensity of the fluorescent light. Exposures may be prolonged provided that irradiation does not interfere with fluorescence. The earlier alleged fluorescence photomicrographs in transmitted light (Haitinger, 1930; Vodrazka, 1930 *b*) have been shown by Metzner (1930 *a*) to present only pictures due to the back-reflected ultra-violet light. With the modern photographic emulsions which are highly sensitive to the whole spectral range, perfect fluorescence photomicrographs can be obtained [even coloured ones (Grabner, 1933)] without difficulty (Wimmer, 1935; Barnard, 1937). The most important condition is the use of filters sufficient to remove all traces of the primary radiation.

The photography of the intravital microscopic image is a very much more difficult proposition, since the exposure is limited. The use of objectives of great numerical aperture is advisable. At the time of introduction of the method (1929) the existent photographic emulsions were not sufficiently sensitive to green and yellow, the colours predominantly obtained by using fluorescin and acriflavin. The photomicrographs then taken by Ellinger and Hirt proved to be ultra-violet reflex pictures and have not been published. The lack of suitable emulsions, and not as Franke (1935) believes the absence of suitable filters—Ellinger & Hirt (1929 *a*, *b*, 1930 *b*) had already worked with those used by Franke—was the cause of the failure to obtain photomicrographs as early as 1929. Since then, photographic emulsions have been improved so much, both in their uniform sensitivity to the various spectral ranges as well as in their speed, that the reproduction of fluorescent images even of comparatively low intensity has been achieved with the use of adequate trap filters and with short exposures. Such black and white intravital photomicrographs

have been published during recent years (Franke & Sylla, 1933; Ellinger, 1935, 1938, 1940; Hirt, 1939 *a* and others). Franke (1935) has given a summary of the method. It would seem remarkable therefore that in recent papers (Sturm, 1936, 1938) the statement should be made that photographic reproduction of fluorescent images is still impossible. The difficulty in the photomicrography of living organs is now largely due to the difficulty of avoiding pulsation of the organs. Franke (1935) gets out of the difficulty by simple means; he states that the fluorescent picture does not change immediately after death, and when he is unable to fix the living organ sufficiently he contents himself with the photomicrograph of the dead. This conception of Franke is not valid. The fluorescent image due to fluorescin changes very rapidly after death. The study of a physiological process cannot be carried out with the dead organ. By careful procedure it is possible to abolish pulsations of most organs, lung and heart excepted. Series of photomicrographs of the kidney while excreting fluorescin have been published by Ellinger (1940). Actually the photomicrograph can under no conditions replace the visible intravital microscopic observation, since photography shows only a single level, while on visual observation continuous motion of the micrometer screw provides a three-dimensional image. An equivalent reproduction is here only possible when one can so improve the speed of the emulsion as to permit the taking of cinematographs at intervals of less than $\frac{1}{20}$ sec. An increase in the intensity of the fluorescent image can be attained neither by increasing the intensity of the exciting light nor by increasing the amount of injected fluorochroms, since the latter would spoil the differentiation owing to over-irradiation. Improvement in the speed of photographic emulsions is thus the direction in which success might be expected.

Recently, colour films have been produced of such high and uniform sensitivity through the whole of the spectral range, at any rate for small size films, that it has become possible to give reproductions of the microscopic image which are true in colour (Hirt, 1939 *b*; Ellinger, 1939 *a*, *b*). A difficulty in the taking of colour photographs is due to the fact that there exists yet no simple photometer to give information about the intensity of the intravital microscopic fluorescent picture. Owing to the great contrasts in fluorescence microscopy one is readily mistaken about the absolute intensity. For proper reproduction of the colours by the colour film, however, sufficient and accurate exposure is of the greatest importance. If the image and the intensity of illumination do not change quickly one can find the proper time for exposure by testing with a black and white film of the same spectral range sensitivity and adjusting the exposure in proportion to the relative speeds of the two films. It is of special importance when using colour films to have adequate trap filters, since even traces of primary radiation cut out all colours but violet and blue. Colour photomicrography allows to record objectively the colour tint observed. This is particularly important in case of estimation of the *p*H of the tissue from the tint of the fluorescin fluorescence (Ellinger, 1939 *b*).

### (4) Fluorescence spectroscopy and spectrophotometry

Fluorescent substances emit light characteristic in colour of the particular substance. Analysis of the spectral range of the emitted light with a spectroscope permits the identification of the light-emitting substance. This can be carried out microscopically by using an eyepiece spectroscope, provided the illumination of the microscopic image is mainly due to the fluorescence of one fluorescent substance, as is the case with porphyrins or lyochromes. The emission lines or bands observed with a spectral eyepiece then allow of the identification of fluorescent substances present in the tissues. Lehmann (1913) recognized that it might become desirable to determine the spectrum of the fluorescent light in fluorescence microscopy quantitatively. He therefore fitted his microscope with the spectral eyepiece of Abbe or the microspectral photometer of Engelmann (1888). Microspectroscopy in the fluorescence microscopy became of special importance for the identification of porphyrins in the tissue (Derrien, 1924; Derrien & Turchini, 1925). Borst & Königsdörffer (1929) used a universal microspectrograph built according to the specification of Königsdörffer. Also Policard (1925 *b*) describes a method for the quantitative determination of fluorescence spectra in the microscope by means of a prismatic eyepiece, while Dhéré (1933, 1939) gives a summary of his and his pupils' studies concerning fluorescence spectra of biologically important substances.

## III. RESULTS

In recent years fluorescence microscopy has been applied to a great variety of problems. It has found widespread use in the proof of the quality of material, e.g. textiles, paper, etc. Only the results of researches dealing with problems of biological interest will be discussed here. Even so, this account does not profess to be complete.

### (1) Examination of the spontaneous fluorescence of animal and plant tissues

#### (*a*) *General spontaneous fluorescence of animal tissues*

The first investigations concerned themselves with the collection of catalogues giving the colours of spontaneous fluorescence which could be seen on observation of the most divergent objects (Stübel, 1911; de Kowalski, 1911; v. Prowazek, 1914; Klemm, 1917; Kaiserling, 1917, 1921; Arloing *et al.* 1925); later studies concerned the eye (Böck, 1934), the brain (Exner, 1932, 1933, 1934), the cerebrospinal fluid (Exner & Klemperer, 1930), the female genitalia (H. Hauser, 1929), various organs of the human body (Bommer, 1929, 1933), animal tissues (v. Querner, 1933; Hamperl, 1934; Sutro, 1936), and the argentaffin cells of the intestines (Erös, 1932). While most of these researches content themselves with the description of the fluorescent colours observed, v. Prowazek (1914) discusses the cause of their formation, considering them due to physico-chemical conditions at the lipoid-water interface. Bommer (1929) also discusses the cause of different cases of fluorescence.

He tried to extract and identify the fluorescent material from the sections, and succeeded in this to a certain extent with a red fluorescent substance from the follicles of the human face which is probably a porphyrin.

### (*b*) *Porphyrins*

Fluorescence microscopy has proved particularly useful in the investigation of porphyrins. It was a great advance when Derrien and his co-workers (Derrien, 1924; Derrien & Turchini, 1924, 1925) succeeded in demonstrating by fluorescence microscopy the presence of porphyrins in Harder's glands of rats and mice, in the zones of growth and calcification of the bones of young mammals and foetuses, in the incisors of newly born guinea-pigs, and also in the deposits on the tooth-gum edge. These observations have been confirmed and extended (Fikentscher *et al.*, 1931, 1933; Loos, 1931; Pflüger, 1931; Fikentscher, 1935). Very extensive investigations on the distribution of porphyrins in the tissues of a patient who had died of congenital porphyria were made by Borst & Königsdörffer (1929), and corresponding work was done by Fikentscher (1930) on tissues of cattle who had been suffering from ochronosis. Porphyrins in bile, urine and liver cells but not in the kidney were found after drug poisoning (Phanodorm, Sedormid) (Emminger, 1933). Emminger & Battistini (1933) failed to find porphyrins in the kidneys of rabbits suffering from lead-poisoning, though they were plentifully excreted in the urine; the erythroblasts in the bone marrow of these animals were shown to contain and probably also to produce porphyrins. Seggel (1934, 1937) and Keller & Seggel (1934) made the interesting observation that red fluorescent erythrocytes occur in healthy men and in a variety of animals. During certain anaemias these are increased in number, especially during treatment. Their fluorescence is probably due to protoporphyrin, the increase of which, particularly during treatment, has probably to be regarded as a symptom of regeneration. Policard (1924) found a red fluorescence in necrotic tumour tissue which he attributes to porphyrins (see also Körbler, 1931, 1932). Fluorescence microscopy has been used also for the detection of porphyrins in faeces (Urbach, 1938).

### (*c*) *Spontaneous fluorescence of plant tissues*

Spontaneous fluorescence in plants was investigated very early by Klemm (1917) and later by Metzner (1930 *b*), who studied in particular the permeability of plant tissues to long-wave ultra-violet radiation. Spontaneous fluorescence was observed in the internal portion of the bark of living branches which changes when the branches dry up and disappears entirely when they die of cold (Haitinger *et al.*, 1929). Eichler (1934, 1935) studied the formation of wood by noting the changes in the spontaneous fluorescence during its progress. He showed that the spontaneous fluorescence of the wood is due to deposition of lignin or xylin. Investigations regarding the fluorescence of wood have also been performed by Vodrazka (1930 *b*). The very intense fluorescence and the characteristic fluorescence spectrum of chlorophyll was used for its microscopic localization in plant tissue (Tswett, 1911; Wilschke, 1914).

### (*d*) *Lyochromes* (*vitamin* $B_2$, *riboflavin*)

The first investigation with the intravital microscope showed the presence of a yellow-green fluorescent substance in kidney and liver which led to the discovery and isolation of the lyochromes, a new group of natural pigments, by Ellinger & Koschara (1933 *a*, *b*, *c*, 1934). Its most important member was shown to be identical with one constituent of the vitamin $B_2$ complex, lactoflavin; this substance was isolated simultaneously by Kuhn *et al.* (1933). The distribution of lyochromes in the optic nerve, lens and vitreous humour was investigated in frozen sections of eyes of the cod and some mammals by v. Euler *et al.* (1935). In fresh cod eyes it was found in the retinal pigment, whether the eye had been exposed to light or kept in the dark. It diffuses rapidly after death. Intravital microscopic examination of its distribution in the kidney and of the response of the kidney towards injected lactoflavin by Ellinger (1938) showed that it occurs there in two modifications, viz. (*a*) a green fluorescent form in the lumen of the tubules and excreted in the urine, and (*b*) a yellow fluorescent form in the epithelial cells of the proximal tubules, even after greatly prolonged lactoflavin deficiency in the diet these yellow fluorescent particles are hardly diminished. Lactoflavin elimination by the kidney corresponds to that of fluorescin. The localization could be shown photographically (Ellinger, 1938). Hirt (1939 *a*) and Hirt & Wimmer (1939 *a*) have investigated its distribution and excretion in the liver, and have shown that it behaves here also like fluorescin. They observed in the liver and other tissues after treatment of animals with acriflavin red fluorescent particles which they ascribed to a hypothetical storage form of the complex vitamin $B_2$ and also to the presence of heparin. In a later paper, Hirt (1939 *b*) stated that this red fluorescence can only be obtained with one certain preparation of acriflavin. This appears to be altogether unintelligible, since acriflavin is a single chemically well-defined substance. When the effect can be obtained only with a certain acriflavin preparation it must be due to an impurity present in this preparation. Ellinger (1939 *c*), at any rate, has not been able to confirm the observation of Hirt dealing with the occurrence of red fluorescence in the liver with a preparation of acriflavin highly purified by a number of recrystallizations.

### (*e*) *Vitamin A*

v. Querner (1932, 1935) and v. Querner & Sturm (1934) observed a very strong spontaneous fluorescence of the isotropic lipoid droplets in liver cells when investigating unstained liver sections. The fluorescence was destroyed rapidly by ultra-violet and more slowly by white light, as also by treating the sections with alcohol, chloroform, acetone, but not with water, glycerol or formalin. In transmitted white light these fat droplets did not differ from normal fat. Fluorescent material with the same properties was also found in retina, adrenal medulla, anterior lobe of the pituitary (basophile cells), and in fresh liver oils, cream and butter. It was found to be reduced in rats which had been fed on a vitamin A-free diet. By analogy it has been regarded as vitamin A, and its formation in the liver is regarded as likely. The experiments have been repeated by Hirt (1939 *a*). After feeding

large quantities of vitamin A (preparations not described) to mice their organs were investigated and liver and lung were found to contain vast numbers of golden yellow fluorescent granules mostly localized in the endothelia of the vessels and only sparsely in the liver cells, especially in the pigment cells. Hirt (1939*a*) came to the conclusion that the fluorescent substance is undoubtedly vitamin A and that the storage of vitamin A in Kuppfer's star cells is thus proved. The difference in the localization observed by v. Querner and by Hirt is ascribed by the latter to differences in the experimental conditions. A bright fluorescence ascribed to vitamin A was observed in the pigment cells of the retina of rats adapted to light while those of animals kept in the dark showed no fluorescence. The intensity of the fluorescence could be increased by feeding carotene or vogan (v. Jancsó & v. Jancsó, 1936).

(*f*) *Other vitamins*

In a further communication Hirt & Wimmer (1939 *b*) believe they can observe the localization of nicotinic acid and nicotin amide in the tissues and the effects of its injection by fluorescence microscopy. They claim that in a 1 % aqueous solution the former shows weakly yellow, the latter more strongly yellow fluorescence. Ellinger (1939 *c*) has not been able to observe such fluorescence in aqueous solutions of highly purified preparations of both substances and cannot agree with the conclusions drawn by Hirt & Wimmer, nor can he accept their statement that vitamin C can be observed microscopically by its fluorescent properties.

(2) FLUORESCENCE MICROSCOPY IN BACTERIOLOGY

In spite of very detailed descriptions given in numerous publications by Haitinger and his co-workers (Haitinger, 1934, 1935, 1938 *a*, *b*; Haitinger & Exner, 1936; Haitinger & Hamperl, 1933; Haitinger & Linsbauer, 1933, 1935; Hamperl, 1934; Exner, 1937) for the preparation of animal and plant tissue for fluorescence microscopy especially by devising numerous fluorescent dyes for the staining of various kinds of tissues, no spectacular results have been obtained so far by fluorescence microscopy in transmitted light in animal or plant histology. A considerable amount of useful work, however, has been carried out by fluorescence microscopy in transmitted light in bacteriology. In bacteriology, fluorescence microscopy appears to have been first applied by Kaiserling (1917, 1921) who only describes and notes the spontaneous fluorescence of a few bacteria probably expecting to find some specific differences and to use these to build up a diagnostic method. Human and bovine tubercle bacilli are said to fluoresce in different colours. Similar investigations were also made by Arloing *et al.* (1925). Barnard & Welch (1936) showed in comparative investigations both on light and dark ground that parts of the bacterial cell body but not the cell membranes fluoresce. Barnard (1937) examined the virus bodies of foot-and-mouth disease and vesicular stomatitis with the fluorescence microscope in transmitted ultra-violet light and with ultra-violet dark-ground illumination. The results are illustrated by perfect photomicrographs. The size of the virus bodies revealed by microscopic measurements was in remarkably good agreement with that found by filtration experiments.

v. Jancsó (1932) succeeded in observing the action of fluorescent chemo-therapeutic substances (acriflavin and others) on trypanosomes. The drugs were injected into the infected mice and rats. In the blood picture of the treated animals the parasites showed intense fluorescence. Strains, however, which were resistant either to arsenic or acriflavin remained unstained, so that it was proved that these drugs have to penetrate into the parasites in order to become effective. Corresponding experiments are also described by Fischl & Schwenk (1932), and Fischl & Singer (1935) were able to localize also atebrin in nagana and bird-malaria parasites, while Bock & Oesterlin (1939) succeeded in making visible the storage of atebrin and quinine in the parasites of monkey malaria. There is no doubt that by his observations on the effect of chemo-therapeutic drugs on parasites, v. Jancsó has opened a valuable new field to fluorescence microscopy.

Staining of bacteria and protozoa which is otherwise difficult promises success with fluorescent substances and the use of a fluorescence microscope. Hagemann (1937 *b*) recommends berberin sulphate for staining tubercle and lepra bacilli, Hagemann (1938) and Keller (1938) auramin for the former which is also described by Bock & Oesterlin (1939) for the same purpose. Herrmann (1938) confirms Hagemann's results on tubercle bacilli and claims an improvement of the auramin staining. Bock & Oesterlin (1939) were able to stain a large number of protozoa with rivanol and primulin. Finally, Hagemann (1937 *a*, *c*) claims the successful selective staining of certain viruses with primulin so that they become visible in the fluorescence microscope at a magnification as low as × 200. For certain kinds of viruses Hagemann's observations have been confirmed by Wolfram (1938) [*Pemphigus vulgaris* and *Dermatitis herpetiformis*] and by Bock & Oesterlin (1939), but the method has failed in the presence of lipoid or fat material. For Gerlach's (1937 *a*, *b*, 1938 *a*, *b*) claim for a successful fluorescence microscopic demonstration of elementary bodies from tumours and of the virus of the foot-and-mouth disease by means of Hagemann's method, one has to wait for confirmation owing to the apparent lack of adequate controls in these experiments.

### (3) Results of intravital microscopic studies

Intravital microscopy naturally found scope for its employment in the observation of resorptive and excretory functions of different organs and its most important fields of research are the glands and the body fluids. The leading idea, however, which led to the development of the method was the desire to obtain information about the physical and physico-chemical conditions and processes in the cell and in particular about the electrostatic phenomena (Ellinger, 1929). For this purpose fluorescin proved itself to be the ideal indicator. Besides, the method had given useful help in the elucidation of numerous and quite divergent problems. It has been successfully used for instance for the observation and measurement of the width of blood vessels (Ellinger & Schmitt, 1933), which permitted the investigation of the mode of action of capillary poisons and a study of the permeability of the capillary walls allowed Eppinger and his co-workers (Eppinger, 1937; Kaunitz & Schober, 1937; Roller & Schober, 1937) to make detailed investigations on serious

inflammations, while Vanotti & Pfisterer (1933) compared the amount of capillaries in trained and untrained muscles.

There exists a definite danger in using the method of intravital microscopy. The pictures gained with the method differ entirely from those gained by observing sections with the usual microscopy in transmitted white light. The interpretation of the pictures is therefore difficult, and frequently the evidence is insufficient for a satisfactory interpretation to be made. In these cases it is advisable to concern oneself with the description of the picture only and to supplement subjective observation by photography wherever possible.

### (a) *Examination of animal objects*

In the first papers by Ellinger & Hirt (1929 *a*, *b*, 1930 *b*) observations on different organs: kidney, liver, adrenal, testes, thyroid, lung, pancreas, stomach and intestinal tract, skin, central nervous system and peripheral nerves of various animals by intravital microscopy were described. In addition to the above-mentioned investigations of spontaneous fluorescence of kidney and liver the functions of various organs have been investigated.

The function of the kidney was studied by observation of the elimination of fluorescin, acriflavin and acid under normal and pathological conditions in the frog by Ellinger & Hirt (1929 *c*, 1930 *a*, 1931) and in the rat by Ellinger (1934 *a*, *b*, *c*, 1935, 1940). It was possible by this method to confirm the experiments of Nussbaum and of Ghiron which are of great importance for the theory of urine formation, and to elucidate their interpretation which has long been a controversial matter (Ellinger 1934 *a*, *b*, *c*, 1935, 1940). The presence of an excretory function of the proximal tubules was also demonstrated (Ellinger, 1934 *a*, *b*, *c*, 1935, 1940). The acidification of urine in the frog's and the rat's kidney was found to occur in the proximal as well as in the distal tubules if the urine was strongly acid and almost exclusively in the latter if the urine was slightly acid. These results were recorded by colour photomicrographs of the tint of the fluorescin fluorescence in the tubular lumen (Ellinger, 1939 *b*). The mechanism of the mode of action of phlorrhidzine has been similarly cleared up by Ellinger & Lambrechts (1937 *a*, *b*, *c*). The observations by Ellinger & Hirt dealing with fluorescin elimination have been confirmed by Singer (1933 *a*) and supplemented by observations on the elimination of aesculin. Observations by Singer (1933 *a*) on the excretion of acriflavin, which differ from those of Ellinger & Hirt, are probably due to the use of an ineffective microscope. More recent studies by Singer (1936 *a*) on the function of the Malpighian body of the frog's kidney are partly based on the obviously wrong supposition that the fluorescence of fluorescin is yellow in the alkaline and green in the acid region. They are therefore valueless. The same holds good for the intravital microscopic part of the publication of Keller & Singer (1939) on the role of the electrical potentials of cells and tissue fluids. Experiments on the excretion of fluorescin on *Daphnia* were made with imperfect technique by Sturm (1936). The same can be said of the observations on the kidney function of frogs after chloroform poisoning or choledochal ligature by Hartoch (1931).

In addition to the function of the kidney that of the liver has been investigated most fully. The excretion of fluorescin in the frog's liver has been shortly described by Ellinger & Hirt (1929 *a*, *b*, 1930 *b*). A few observations of the same process dealing with the frog's liver under normal conditions after poisoning with chloroform, ligature of the choledochus, or irradiation with X-rays have been made by Hartoch and his co-workers (Hartoch, 1931; Hartoch & Israelski, 1932), but cannot be regarded as satisfactory. Franke & Sylla (1933) have investigated very exhaustively the excretion of fluorescin by the normal frog's liver. They described also the mode of action of different blood poisons and of chloroform on the liver function, and supplemented their experiments by the first good photomicrographs. The investigations on the function of the normal frog's liver were confirmed and extended by observations on the response of the rat's liver to fluorescin and acriflavin by Hirt and his co-workers (Hirt, 1934; Ansorge, 1934; Markstahler, 1934; Hirt *et al.* 1939). Sturm's (1938) experiments on the bile capillaries of Salamander larva are open to serious objections on account of the method used.

Excretion and absorption of dyestuffs by the epithelium of the frog's stomach were examined by Henning (1932 *a*, *b*), and valuable results were obtained from a study of the mechanism of secretion and excretion on the one hand and that of resorption on the other. Results, reported by Pick & Zuckerkandl (1935) on differences in capacity of various fluorescin salts to be absorbed by the intestines of frogs are unlikely.

May (1934) succeeded in obtaining results of importance for lung pathology by observing the nature of circulation in the blown and normal lung and Pfaff and his co-workers (Pfaff & Herold, 1936 *a*, *b*, 1937; Pfaff, 1937) could show in rabbits experimentally infected with tubercle bacilli that the central part of the infected lung is supplied with blood vessels from the very beginning of necrosis up to the complete destruction or calcification.

Investigations on the thyroid gland of the rat have been made by Hartoch (1932, 1933) and by Peczenik (1935). They deal with the nature of the secretion of colloid and with the behaviour of the blood supply under the influence of different drugs and the action of the nerves. Corresponding observations have been made by v. Hattingberg & Hartoch (1933) on the function of the maxillary salivary gland of the grass snake. Singer & Zwemer (1934) describe the appearance of the frog's adrenal gland in both back reflected and fluorescent light and claim to have observed some changes after pithing or the administration of curare or mercury chloride.

Singer (1933 *b*) has investigated the leucophore network of the frog's skin whose function has been said to be that of a light filter. Sturm (1938) has described the staining of the epithelia of the Salamander's skin by fluorescin and acriflavin, and Hirt (1939 *a*) in particular has concerned himself with the mast cells of the subcutaneous connective tissue of the mouse. The remarkable red fluorescence of the latter after injection of acriflavin has led to far-reaching conclusions regarding their function.

The microscopical observation of the function of the eye has long held a place of its own because its position and arrangement render it particularly suitable for

such studies. It was the first living organ used for fluorescence microscopical studies, if only with weak magnifications (corneal microscope), by Thiel (1926). The permeability and the staining of its membranes have been investigated exhaustively for the most divergent vital dyes by Fischer (1929 *a*, *b*, 1930).

### (*b*) *Examination of plant objects*

Intravital microscopy appears to be particularly suited for the solution of problems in plant physiology because, first, plant objects need comparatively little preparation for observation owing to the large surfaces and small depths of many plant organs, so that the whole organ can be examined, and secondly, because the fixing of the position of the organ, so often difficult in animals, is unnecessary. It is therefore astonishing that the method has been only rarely applied up to the present. Valuable results dealing with absorption, storage and circulation of fluorescent dyestuffs in plant tissues have been gained by the investigations of Schumacher (1933, 1936, 1937), Döring (1935) and Strugger (1938 *b*, *c*) which have been in part illustrated by excellent photographs. The former have been criticized by Rhodes (1937) who was unable to trace fluorescin in tissues at *p*H lower than 5·0.

## IV. SUMMARY

1. Fluorescence microscopy is based on the principle of illuminating the microscopic object by fluorescent light produced in the object itself. This must either contain fluorescent pigments or must be prepared for the purpose by a previous injection of fluorescent dyes. The fluorescence is excited by light rays of short wave-lengths which are focused on to the object from a special source of light. Fluorescence microscopy can often be employed most fruitfully where microscopy in ordinary white light is inapplicable, particularly in the following fields of research: (*a*) for the detection of spontaneously fluorescent pigments and the examination of their distribution in the tissues in translucent and opaque objects; (*b*) for translucent objects treated with fluorescent dyestuffs, in which case fluorescence microscopy may render visible structures which cannot be observed in white light; (*c*) for the microscopic investigation of processes in living organisms which have previously been injected with suitable fluorescent dyes (intravital microscopy).

2. Two fundamentally different types of fluorescence microscopes are in use, one using transmitted light for translucent objects, the other using incident light for opaque objects. As primary source of light lamps rich in light of 300–400 m$\mu$ and of great intrinsic brightness are used. The light beam is cooled by water and is then freed from the visible and infra-red rays by suitable filters.

For fluorescence microscopy in transmitted light the ultra-violet beam is collected by a quartz condenser and projected on to a totally reflecting quartz prism which deflects the beam into a quartz Abbe condenser fixed in the substage of an ordinary microscope.

For fluorescence microscopy in incident light two types of illumination are used: inside and outside illumination. In the former the microscope tube is fitted with a side tube through which the ultra-violet beam is projected by a quartz condenser

on to a totally reflecting prism or plate. These are fixed in the microscope tube so that they deflect the beam into the objective which acts simultaneously as condenser. In the outside illumination type the ultra-violet beam is focused on to the object either unilaterally by lenses or mirrors or from all sides by mirrors. For fluorescence microscopy in incident light, stands with vertically movable stages are essential. For the inside illumination type, objectives are made from glass readily permeable to wave-lengths of 300–400 m$\mu$. For all types, objectives must be free from fluorescent material. For intravital microscopy only water immersions are used, which are constantly irrigated with a physiological salt solution. In all types trap filters have to be used to keep away from the eye any traces of the primary light.

3. For fluorescence microscopy in transmitted light the object must be prepared without using any fluorescent mounting material. Objects which do not contain fluorescent pigments have to be treated with fluorescent dyes, a large number of which has been described for this purpose.

For intravital microscopy animals must also be treated with fluorescent dyes, fluorescin and acriflavin being most suitable. The former allows a simultaneous estimation of the *p*H of the tissue.

4. Photomicrographs of the fluorescent image in black and white as well as in colour can be obtained both from inanimate and from living objects. In the latter case the correct fixation of the object is the most difficult problem. Much care is also necessary to prevent the slightest trace of the primary light passing into the camera.

5. By using a spectral eyepiece it is possible to identify a fluorescent pigment present in the tissue by examination of the emission bands of the fluorescent light.

6. The fluorescence microscopic examination of unstained objects has led to the detection and isolation of natural fluorescent pigments, as in the case of lyochromes (riboflavin, vitamin $B_2$), and to the observation of the distribution of fluorescent pigments in animal and plant tissues (chlorophyll, porphyrins, lyochromes, vitamin A).

No results of great importance have so far been obtained by applying fluorescence microscopy to normal or pathological tissues stained with fluorescent dyes.

Valuable results have been gained by fluorescence microscopy in the study of the mechanism of the effect of fluorescent chemotherapeutics on parasites and in bacteriology. The study of virus bodies might become a particular successful field for the method.

Intravital microscopy has been successfully applied to the investigation of the physiology and pathology of animal and plant organs, particularly in dealing with the function of various glands and body fluids.

7. Fluorescence microscopy of tissue sections, smears, or other translucent preparations has two primary purposes:

(*a*) In unstained preparations it allows of the recognition and localization in animal and plant tissues of spontaneously fluorescent substances which cannot be discovered by any other methods. Here the method will probably have a great future.

(*b*) In preparations treated with fluorochroms it is a new method of staining, the value of which is, however, not greater than that of other staining methods, though it has led to important results in special cases, as in the localization of chemotherapeutics acting on parasites.

In intravital microscopy which uses fluorescent microscopy only as a means to an end, we have, however, a method which has opened a new field for research, as it permits the observation of biological processes occurring within living organs and cells by extending the two-dimensional pictures of the usual microscopy into three dimensional space and adding the fourth dimension of time. The results gained up to now should represent only the beginning of a new and promising field for research.

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## ADDENDUM

The sources of light have been augmented by the design of a small high pressure mercury lamp, Lux u.w., suitable for bacteriological investigations, in addition to the larger Lux u.v. (Keller, 1939; Melczer & Venkei, 1940). The theory and the available systems of illuminating apparatus for transmitted and particularly for incident light have been exhaustively and critically described by Hauser (1939 *a*, *b*), while a short review of ultra-violet microscopy has been published by Caspersson (1939); the most recent developments of the luminescence microscopy have been described in a pamphlet of Zeiss (1940).

Spontaneous fluorescence has been observed in the silk glands of the silk moth (Policard & Paillot, 1925; Beer, 1928, 1930); in those of spiders (Turchini & Millot, 1926), in the peritrophic membrane of the intestines of orthopterans (Salfi, 1937); a green fluorescent pigment has been observed by Simonetti (1936) in the integument of the latter.

A greenish fluorescence has been found in various plants after treatment with alkali by Klein & Linser (1930), who have also examined the distribution of the fluorescent esculin in *Aesculus hippocastanum* (1930, 1932).

Popper (1940 *a*) has studied by means of fluorescence microscopy the localization of vitamin A in the liver and the epithelium of the adrenal cortex in normal rats, rats deficient in vitamin A, in the latter after adding vitamin A to the food, and in livers of rabbits, monkeys, dogs, guinea-pigs, mice and frogs. The experiments have however, been carried out with less care and inferior methods than those by v. Querner (1932, 1935) and by Hirt (Hirt *et al.* 1939) on the same subject. Popper (1940 *b*) has also examined vitamin A in livers, adrenals, and other organs of human beings. He claims that in infectious diseases the number of green fluorescent Kuppfer's star cells is increased and in exhausting diseases, acute hepatitis and cirrhosis diminished.

Wimmer (1939) describes changes in the spontaneous fluorescence and in the red fluorescence following the injection of Hirt's "special acriflavin" after reduction or increase in the diet of vitamin A, $B_1$, $B_2$, C or nicotinic acid in various organs of frogs, mice and rats. He concludes from the results that the reticular endothelial system plays an important rôle in the vitamin metabolism and he draws far-reaching conclusions concerning the vitamin therapy.

Hagemann's results on the successful staining of tubercle bacilli with auramin have been further confirmed by Clauberg (1939), Dabelstein (1939), Didion (1939) and Küster (1939). The latter recommends auramin also for the staining of diphtheria bacteria while Keller (1939) uses coriphosphin H for this purpose. Acridin-yellow is recommended for the staining of tubercle bacilli by Haitinger & Schwertner (1939). Hagemann (1939) claims that the auramin staining of tubercle bacilli provides by far the most effective diagnostic method. He considers the fluorescence microscopic reproduction of the virus elementary bodies to be not real images but due to deflexion phenomena. By treatment for 15 sec. with aqueous solutions of phenol and primulin virus up to 100 m$\mu$ can be observed as blue white

fluorescent spots. As this staining is destroyed by irradiation it is preferable to use resorcin-thioflavin which produces a yellow brown fluorescence of the elementary bodies of smallpox, ectromelia and molluscum contagiosum. Magnifications up to 4000 × can be used.

Jaeger & Jaeger (1939) make use of the fluorescence microscope for the examination of the surface of the human skin previously stained with primulin or auramin. Hirt (1939 *c*) has given a survey of the use of fluorescence microscopy in medical research, and has (Hirt 1939 *d*) introduced his "special acriflavin" for the fluorescence microscopic reproduction of unmyelinited nerves, showing a red fluorescence which becomes brown yellow by irradiation. This staining is attributed to the fixation of the dye to riboflavin. Myelinited fibres remain unstained, the axis cylinders fluoresce weakly green, the myelin sheath light yellow. Cerebrum and cerebellum fluoresce green from free riboflavin. This fluorescence is increased after addition of riboflavin to the food.

A survey of the use of fluorescence microscopy in botanical research has been given by Strugger (1939 *a*) who has also continued his studies (1939 *b*, 1940) on the adsorption, storage, and circulation of fluorescent dyestuffs in the plant. He has examined the behaviour of berberin sulphate, eosin, sulphorhodamin G and oxypyrentrisulphonic acid in the plant. The latter like berberin was found to be diphasic, the yellow fluorescent coarsely dispersed and the blue fluorescent finely dispersed particles moving in different channels in the plant, as was the case with rhodamine BS in the experiments on animals of Ellinger & Lambrechts (1937 *c*). v. Loui (1930) has used the fluorescence microscopy for a study of the individuality of the plastides and Johannes (1939) has examined the staining of the mycelium of fungi with berberin and fluorescein. From this staining the isoelectric point of the plasma and of the central body of the nucleus has been estimated.

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# QUANTITATIVE CHANGES IN PIGMENTATION, RESULTING FROM VISUAL STIMULI IN FISHES AND AMPHIBIA[1]

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(*Received* 15 *January* 1940)

CONTENTS



## I. INTRODUCTION

It is hardly necessary at this time to state that the familiar rather rapid colour changes which are undergone by various cold-blooded animals are not due to changes in the amount of pigment present. Even the most striking of such changes result from shifts in the position of pigment particles which do not vary in amount from moment to moment. It is pretty generally agreed, furthermore, that this rearrangement of pigment matter takes place within the limits of cells which maintain nearly constant outlines. Amoeboid movements of the chromatophores are believed to play a minor role, if any, at least in adult animals.

It has for some time been recognized, however, that there are other, more enduring changes of colour or shade which are due to actual changes (increase or decrease) in the amount of pigment in the tissues of the organism. These have been designated as "morphological" colour changes, in contradistinction to the former or "physiological" type of changes. It is clear that these terms are not very appropriate, inasmuch as both of these processes are equally *physiological*. It seems hardly practicable to dispense with them altogether, however, owing to the lack of a suitable pair of antonyms. I have, nevertheless, wherever possible, substituted "transitory" for "physiological" and "quantitative" for "morphological". It is interesting that a vast majority of the published papers in the field of colour change

[1] Contributions from the Scripps Institution of Oceanography, New Series, No. 98. Assistance is acknowledged from W.P.A. Project 665-03-3-141.

have dealt with these immediate transitory or "physiological" responses of the chromatophores. Indeed, Fuchs, in his monumental review of colour change in animals (1914), appears to question the existence of quantitative ("morphological") colour change in response to optical stimuli, and von Buddenbrock, as late as 1928, was aware of no convincing evidence for fishes.

The present review will be concerned with quantitative changes only, except in so far as transitory changes are believed to be causally related to these. Under "quantitative" changes are here included any changes in the amount of pigment whether or not these have been submitted to actual measurement. This review will further be restricted to such pigmental changes as result from visual stimuli, or the withdrawal of these. The effects upon pigmentation of various other physical and chemical stimuli and of food cannot be considered here. Nor can the numerous and important studies of invertebrate animals be included.

For the purposes of the present discussion, the visual (optic) environment must be divided into (1) incident light and (2) the background. Such a division may seem to be an altogether artificial one, since most organisms can hardly be supposed to recognize the distinction between the source of light and the light which is reflected from surrounding objects and the substratum. However, the difference is of vital importance. Poikilochromic[1] animals respond in a high degree to differences in the shade or colour of the background, much less to differences in the intensity of the incident light. This is not at all mysterious when we recognize that the distinction here drawn is altogether a topographic one. The "background" comprises that part of the visual field which lies below a certain level, while "incident light" comprises that part which lies above that level. The contrasted effects of these two portions of the visual field depend, as will be pointed out below, upon functional differences in the upper and lower halves of the retina.

So far as shade (paleness or darkness) is concerned, the response of an animal to its background is a response to the latter's albedo. By albedo is here meant the proportion of incident light which is reflected or dispersed from a given surface. It is what makes the difference, for example, between white and a neutral grey. Thus the pigmentary response, whether "physiological" or "morphological", is largely independent of the absolute degree of illumination. It is not, however, wholly independent of this.

In the two preceding paragraphs it was assumed that we were dealing with white light. Illumination by limited portions of the spectrum have, however, been employed by a number of investigators, and have been found to exert specific effects upon the chromatophores of some organisms, and even upon pigment formation. In these cases, the quality of the incident light has been effective, quite independently of the background.

Numerous other experiments have been performed which deal with special modifications of the visual field: inverted illumination, total darkness, blinding, etc.

Investigations of chromatophore responses have had to do chiefly with the

[1] This term, which I have already employed (1935), seems a legitimate analogue of such terms as poikilothermic.

black cells (melanophores); to a far less extent with the white cells (guanophores) or the coloured cells (lipophores). This is because the melanophores are far more conspicuous than the other types of chromatophores, and are far more resistant to fixing and clearing reagents. Some important observations have, however, been made upon both guanophores (iridocytes) and lipophores. Quantitative determinations, in the case of melanophores, have consisted in either counting the actual number of these cells in a selected area of the skin, or in assaying the amount of melanin in the body as a whole. Both of these procedures have likewise been employed in the case of lipophores (at least of xanthopores). So far as I know, no precise determinations have been reported for guanophores.

Some of the phenomena here considered raise questions of great interest in the field of theoretical biology. Considerable portions of this review must accordingly be devoted to the discussion of such questions.

## II. EFFECTS OF BACKGROUND UPON THE FORMATION OR LOSS OF PIGMENT

### (1) *Melanin*

So far as I know, Flemming (1882, cited in 1897) was the first to point out that the shade of the background might be influential in modifying the quantity of pigment in an animal. The experiment to which Flemming refers was concerned with the effect of a white background upon the pigment of salamander larvae. He does not seem to be altogether certain, however, as to the nature of the effective stimulus.

Šecérov (1909) exposed the loach, *Nemacheilus barbatulus*, to various backgrounds, coloured and uncoloured, apparently with pronounced results upon the quantity of visible pigment. Some of this writer's claims are incredible, however (see below), a circumstance which must be borne in mind in evaluating his work. Šecérov seems to have been the first to employ the terms "physiological" and "morphological" colour change, and was perhaps the first to recognize the distinction between the classes of phenomena thus designated.

Franz (1910), experimenting upon young plaice, noted that these acquired chromatophores when they left off swimming and took up life upon the bottom. He gave reasons for believing that this was due to stimuli from the bottom, and not merely to an advance in the stage of development. Franz's interpretation of these facts is, however, quite untenable.

Babák (1910, 1912, 1913) made the first substantial contribution to our knowledge of the quantitative effects of visual stimuli upon pigmentation in vertebrates, and the mechanism by which these effects are produced. This investigator experimented with *Amblystoma* larvae, of which normal individuals were reared upon white and black backgrounds, blinded ones in the light and in darkness. Some of these were kept nearly six months, the number of chromatophores in several regions of the body being counted at intervals. He reports that at the end of this period an individual on black had nearly twice as many chromatophores as one on white. The increase in number per week was forty for the "black" animal, eleven for the "white" one. In this case, therefore, there was no absolute decrease in the

"white" specimen, but only a marked retardation in the rate of increase. Elsewhere he reports an actual reduction in the number of chromatophores previously present. Babák also points out that aside from these changes in number there may be a marked increase or decrease in the melanin content of the individual cells.

"There is thus no doubt", asserts Babák (1913), "that the nervous system, under the influence of the light relations of the environment, controls not only the movements and the mass of the pigment in the chromatophores, but also influences the division or multiplication of the chromatophores" (*trans.*).

Kuntz (1917) contributed a valuable paper on "The histological basis of adaptive shades and colours in the flounder, *Paralichthys albiguttus*". He reports a reduction of approximately 30% in the number of melanophores in the superficial layer of the dermis when specimens which had been kept for some time on a white background were compared with freshly caught specimens or ones from a black background. This reduction was not so great on a yellow background as upon a white one. It was apparently due to the ejection of melanophores through the skin, a process which takes place normally, though more slowly, in all fishes, regardless of background. A graph which Kuntz presents shows both a decrease in fishes transferred to white backgrounds and an increase when specimens were returned to black. The maximum effect, he found, was attained in 11 days.

The massive contributions of Murisier (1920–1) must be given prominence in any discussion of our present topic. This investigator, working on young trout, found that white and black backgrounds strongly influenced the quantity of melanin produced, without appreciably influencing the growth of the fish. For one experiment, he reports that the mean number of melanophores counted in the dorsal fins of the white-adapted fishes was 295, that for the black-adapted fishes being 680, and for ones kept in total darkness, 330. Corresponding figures for the anal fins were 4, 67 and 5. Murisier believed, however, that whereas sojourn on black leads to a great increase in the number of melanophores, sojourn on white merely causes a retardation of the further production of these cells, not a loss of those previously present. This last conclusion is not in agreement with the findings of most others who have performed similar experiments upon fishes.

The work of Kudô (1922) is sometimes seriously cited in discussions of quantitative pigment changes in fishes. His report as a whole is so unconvincing, however, and his claims so incredible, that I cannot attribute any importance to them. His thesis is that the darkening of a fish, however called forth and apparently of however brief duration, results from an actual increase in the amount of melanin present. Even "Tötung durch Schläge auf den Kopf" produces an increase in this substance. Pictures of test-tubes containing various amounts of dark precipitates are offered as evidence for these effects.[1]

Hewer (1927, 1931), working upon English flatfishes, found that an increase in epidermal melanophores occurred as a result of exposing his fishes for two or three

[1] In a previous paper (1939*a*), I referred to these pictures as not being photographs. I had overlooked a statement in the text: "Die Abbildungen sind direkte Aufnahmen auf Entwicklungspapier, ohne Negativ." Regardless of the process of reproduction of these figures, however, we may doubt whether they are likely to be convincing to anyone who has undertaken the assay of melanin.

weeks to a black background. The numbers both of erythrophores and iridiophores (guanophores) were likewise found to be influenced by the background, the former undergoing an increase on orange and a decrease on white; the latter undergoing an increase on white. Hewer expressed the belief that changes produced in this way through the influence of optic stimuli have a possible evolutionary significance, so that "factors in the environment producing temporary adjustments also produce more permanent effects of a strictly structural character" (1931).

Vilter (1931 *a*) reports an experiment in which he endeavoured to assay the melanin content of two axolotls which had been kept on black and white background for a period of 17 months. The method of Piettre (1911) was employed. This consists in grinding up the tissues in a mortar, mixing them with large quantities of water, allowing the debris to settle, decanting, and then centrifuging the overlying liquid, after this has been kept 24 hr. at a low temperature. Vilter states that four times as much melanin was extracted from the black specimen as from the pale one, and offers a photograph showing the two lots of material. Although this method would seem to be far from precisely quantitative, the difference in the yield of the two individuals appears to be sufficiently marked. It is unfortunate, however, that Vilter did not employ larger numbers of specimens in this important experiment.

For some years, the present writer and several collaborators have been experimenting upon the effects of visual stimuli on the production or loss of pigment in certain fishes. It must be explained that our procedures have been changed from time to time, in the course of these experiments, as likewise our mode of presenting the same data to the reader. For this reason, inconsistencies (more apparent than real) would be evident to a reader who should carefully compare the various reports of this work.

In *Lebistes* (Sumner & Wells, 1933) a rather rapid loss of melanin was found to occur, when fishes of "black" history were transferred to a white background. After a few days, it required but low magnification to reveal the presence of numerous degenerating melanophores in the surface of the skin, while in sections, large rounded masses of melanin were seen to be passing to the exterior through the epidermis. This bleaching process lasted for some weeks. Returning such bleached fishes to black containers resulted, in a few days, in the appearance of numerous small pigment masses in the dermis, which increased in number and size for some weeks. These changes were not confined to the skin, however, for wide differences were found in the peritoneum and other regions between individuals of black and white history, as had already been noted by Murisier (1920–1) for the trout and Vilter (1931 *b*) for the axolotl. (Vilter reports that the livers of black axolotls contain "enormous" quantities of melanin.)

Even the most prolonged exposure to a given background failed to render the resulting state of the chromatophores permanent. Fishes reared from birth in black or white containers, even when the light was continued night and day, and throughout their entire lives, responded fairly promptly to a reversal of their backgrounds, though not so promptly as ones which had been kept for only a month

upon a given background, after attainment of maturity. This was true, indeed, of some which represented a third generation reared under the conditions stated. These last experiments relate to transitory colour changes, but the same statements would doubtless apply to quantitative ones as well.

Odiorne (1933, 1936) experimented extensively in this field at about the same time that our La Jolla experiments were conducted. He employed fishes of a number of species, chiefly members of the genus *Fundulus*. Like several of his predecessors, Odiorne determined, in some cases, the extent of background influence in terms of the density of chromatophore distribution per unit area of skin. It is of interest that while a loss of melanin upon white backgrounds was everywhere manifest (nearly 60% in 10 weeks, in one case), the occurrence of an increase upon black was far less evident. This, it will be recalled, is the reverse of what Murisier found in the case of the trout. There would seem to be nothing inconsistent, however, in the results of these two investigators. The occurrence of a loss or a gain in pigmentation, and the extent of this, might be expected to depend largely upon the background from which a given fish was taken. With some fishes, at least, it is certainly easy to increase the number of chromatophores in a specimen which has previously been white-adapted, and to decrease the number in one which has been dark-adapted. Odiorne, like some others, notes the occurrence (in *Macropodus*) of internal differences in melanophore abundance between dark-adapted and light-adapted specimens.

Thus far, no endeavour seems to have been made to establish any exact quantitative relations between visual stimuli and the ensuing pigmental modifications. Experiments with this in view were commenced in our laboratory, in November 1935. Results thus far obtained have been reported in several papers (Sumner & Doudoroff, 1937, 1938*a*, 1938*b*; Sumner, 1939*a*, 1939*b*, 1940).

In the first series of such experiments, gobies (*Gillichthys mirabilis*) were employed. These were kept in glass aquaria, painted on the outside with black, white and two shades of grey. Sets of aquaria, thus painted, were exposed to two widely different illuminations, these last being approximately in the ratio of 40 : 1.

After the fishes had been submitted to these conditions for nearly three months, an assay of the melanin was undertaken. This consisted essentially of colorimetric determinations of the dark material left in solution after removal of fats, proteins and bone.

These measurements indicate clearly the existence of a graded series of melanin values, corresponding to the series of shades used as backgrounds (white, pale grey, dark grey, black). Moreover, when these values were plotted against the measured albedos of the background, they assumed a logarithmic rather than a linear arrangement.[1] The rule which we proposed provisionally was that the quantity of melanin produced or lost varied inversely as the logarithm of the albedo of the background. On the other hand, it was found that the intensity of the illumination played but a minor part in bringing about pigmental changes, although the more

[1] This was less true of the figures for the more dimly lighted of the two series.

brightly lighted fishes produced slightly more melanin, except in the case of the white (see below).

Similar experiments were later undertaken with *Gambusia affinis*[1] (Sumner & Doudoroff, 1938*a*). These deserve merely passing notice, owing to considerable and quite unaccountable variations in the yield of melanin from lots of fishes of presumably identical history. It is, however, worth noting that the graphs for the background experiment showed a similar trend to that which was encountered in our gobies, i.e. a more nearly logarithmic than linear arrangement of the values. On the other hand, no significant differences were detected as a result of the widely different illuminations employed.

Measurements of melanin were discontinued at this time, and a new technique substituted, namely, the counting of melanophores in given unit areas of the skin. For this purpose, the "guppy" (*Lebistes reticulatus*) was used. The first of these experiments related to the effects of backgrounds. Five of these were employed: white, three shades of grey, and black. Small aquaria of clear glass were used, painted the required shades on the outside. These aquaria were kept in a cabinet divided into four compartments, each lighted by a 100 W. lamp. Readings both of incident light and of reflexion from the bottoms of the aquaria were made with photo-electric equipment.

About thirty-five female guppies were kept in each of these aquaria for a period of about two months. In this, as in all our experiments, the fishes were well fed throughout the period. In the end, they were subjected to adrenalin, in order to bring about the concentrated state of the melanin, necessary for counting the melanophores. After this, they were fixed, and cleared in cedar oil. A slice was removed from the premaxillary region, and the number of melanophores in a given area of definite position was counted with the aid of a Whipple eyepiece micrometer disk.

Fig. 1 represents the frequency distributions for the various melanin values of fifty specimens of each of the five lots of fishes comprised in this experiment. It is probable that the great variability within each of these lots is due largely to genetic differences in a highly mixed stock of *Lebistes*. The mean differences in melanophore number between these various lots, although considerable, are not so great as might have been expected in view of the widely different appearance of these fishes in life. The mean number found in the "black" fishes, for example, is less than three times as great as that for the "white" ones. It is evident that a large part of the visible difference of shade among these fishes is due to factors other than the number of melanophores. Some of it is due to the melanin content of the individual melanophores; much of it, of course, to the state of aggregation or dispersion of the melanin. Fig. 2 is based upon the mean values of the five series represented in Fig. 1. It shows the relations between the number of melanophores per unit area and the albedo of the background on which the fishes were kept. The albedos have been reduced to percentages of the albedo of the white background, taken as 100. The

[1] Owing to misinformation, I referred to this same species earlier (Sumner, 1935) as *Gambusia patruelis*.

numbers given for the melanophores are multiples of the mean number for the "white" fishes taken as unity.

The form of this curve obviously suggests a logarithmic relation between the two sets of values. Fig. 3 shows that this relation holds fairly closely for all of these lots of fishes with the exception of those for black. It would be necessary either to raise the albedo from 0·74 to about 2·0, or to raise the melanophore index from 2·55 to about 3·0 in order to bring the "black" fishes into line.[1] It is plain, nevertheless, that the relation between the two variables, albedo and melanin production, is much more nearly a logarithmic than a linear one. This relationship is of considerable theoretical interest as will be pointed out below.

### (2) *Guanin*

The pigmental effects thus far considered have related solely to the black substance melanin. It has been observed by several investigators (Kuntz, 1917; Murisier, 1920–1; Meyer, 1931; Sumner & Wells, 1933) that decrease in the production of melanophores, in white-adapted fishes, is accompanied by a marked increase in the number of guanophores, or at least in the amount of visible guanin. No quantitative determinations of the latter substance have, however, been reported.

Meyer (1931) states that she undertook such determinations, though she has not indicated her procedure.

### (3) *Carotenoid pigments*

Šecérov (1909) reports "morphological colour changes" in coloured pigments as well as black in *Nemacheilus*. Since, however, he speaks of finding "red, reddish brown, green, greenish, greenish yellow and orange coloured pigments", as well as black and yellow ones, in this fish, his reports regarding pigments must be taken with considerable reservation.

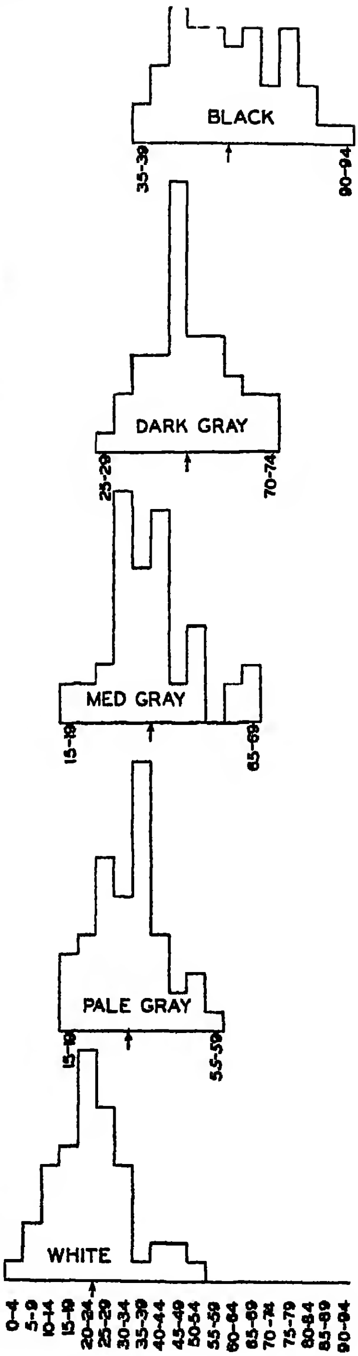


Fig. 1. Frequency distributions of the number of melanophores in a definite area of the premaxillary region of *Lebistes*. Based upon 50 individuals from each of the five backgrounds.

[1] In a recent paper (Sumner, 1939*a*), I provisionally assigned to the black background an estimated value of 2·0, there being, at that time, no available means of determining its exact value. In a graph (Fig. 5) this provisional value was unfortunately included.

Kammerer (1913), after an extended series of experiments, reported not only that young salamanders (*Salamandra maculosa*) responded by quantitative pigmental changes to different backgrounds (black and yellow) but that these changes were ultimately inherited. Even the former of these contentions was disputed by Herbst (1919, 1924), who repeated Kammerer's experiments upon the same species. Herbst reared his specimens from birth to an age of several years. He found that during the larval stages the yellow spots increased in relative size in animals kept upon yellow backgrounds and decreased in those kept upon black ones. After metamorphosis, however, the reverse tendency was manifest, leading to a con-

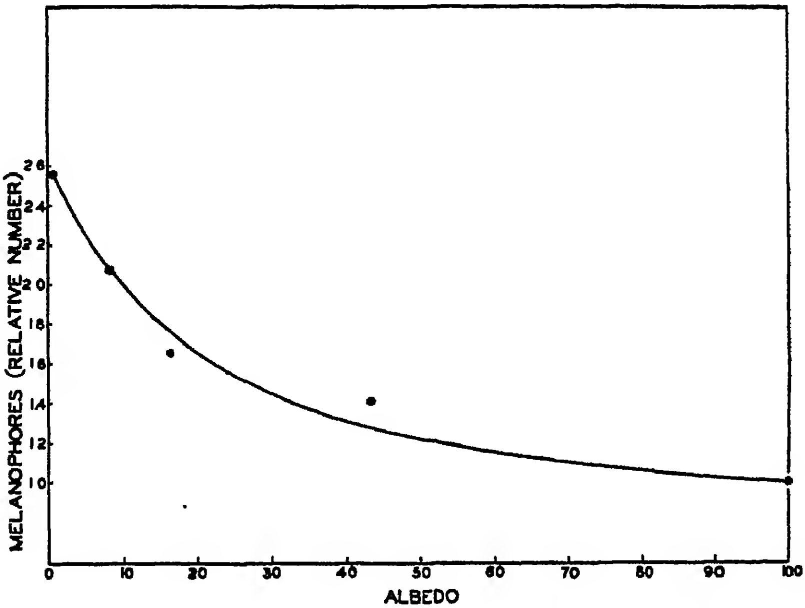


Fig. 2. Relations between albedo and melanophore density, based upon the same individuals as Fig. 1. Abscissas = albedos of the five backgrounds, taken as percentages of that for "white", regarded as 100. Ordinates = mean values for the five lots of fishes, reduced to multiples of the mean value for the "white" fishes.

vergence of the two lots in respect to their colour characters. Since Kammerer started his experiments with post-larval individuals, Herbst believed that he had refuted Kammerer's claims. On the other hand, these claims of Kammerer, as regards the first generation, were confirmed by von Frisch (1920), who concluded that the difference between Kammerer's results and those of Herbst were the results of differing experimental conditions.

Mast (1916), referring to his experiments in which flatfishes were raised on coloured backgrounds, states that "on the reds, greens, and blues adaptive changes still appear to continue after a sojourn on them of between two and three months". Although Mast does not suggest that any quantitative changes in pigmentation occurred on such occasions, the duration of these changes renders it probable that this was the case.

The experiments of Hewer (1927, 1931) may be recalled (see above), since they involve coloured pigmentation as well as black. No determinations appear to have been made by any of these writers, however, either of the number of chromatophores or the quantity of pigment.

The studies of Sumner & Fox (1933, 1935*a*, 1935*b*) relate to quantitative changes in yellow pigment of the xanthophyll series. Fishes of several species (*Fundulus parvipinnis*, *Gillichthys mirabilis*, *Girella nigricans*) were employed in these studies, the xanthophyll being extracted with customary solvents and the resulting solutions assayed colorimetrically. While all these fishes were found to respond in an imitative fashion to yellow backgrounds, as well as to black, grey and white ones, it is of interest that out of the three, *Girella* alone manifested any differences in the actual amount of yellow which it yielded. It is interesting, too, that the amount of xanthophyll present (though not the visible appearance of the fish) appeared to depend upon the shade (whether light or dark), rather than upon the colour (spectral value) of the background. Thus, the greatest amount of xanthophyll was extracted from the almost inky black fishes which had been kept on black, the least from fishes kept on white, while intermediate and nearly equal amounts were yielded by specimens kept on a rather rich yellow and upon a neutral grey of medium shade. It should be added that *Girella*, in all circumstances (though not *Fundulus*), was found to lose xanthophyll under laboratory conditions. The relations stated above indicate, therefore, relative rates of loss.

Incidental, only roughly quantitative, determinations were made of the xanthophyll content of the fishes in certain later experiments (Sumner, 1940). Striking differences were noted in the colour of the 95% alcohol to which the fishes were subjected at one stage of their preparation. In the first of these experiments, in which five backgrounds were used (black, three greys and white), it was obvious that the alcohol from the "black" fishes was by far the most highly coloured (yellow) of any of this series, while that from the "dark grey" ones rated second. The three other lots did not differ appreciably in this respect.

In the second experiment, in which only two types of background (black and medium grey) were employed, the extracts from the "black" fishes were more deeply coloured than those from the "grey" ones at all four intensities of overhead illumination, the latter factor having little or no influence in the matter. In comparisons made with Nessler tubes, it was found necessary to dilute the xanthophyll solution from "black" fishes to 25% of its original strength in order that it should approximately balance that from the "grey" fishes.

In our studies of carotenoid pigments, no attempt was made to count xanthophores, nor were any microscopic studies made. Abramowitz (1935), however, examined and counted xanthophores in the caudal fin of *Fundulus majalis*, before and after keeping the fishes on black, white, yellow and blue backgrounds. After periods ranging from 12 days to 6 weeks, increases were noted on yellow and black, decreases on blue and white.

## III. INTENSITY OF ILLUMINATION

Investigators early discovered that the responses of poikilochromic animals to their backgrounds were, within wide limits, independent of the intensity of the incident light. That this is not absolutely true, however, has been likewise recognized. Brown (1936) has recently shown that at certain very low light intensities, in fishes kept upon identical backgrounds, "the average diameter of pigment masses is directly proportional to the logarithm of the light intensity". Brown was not concerned here with the quantity of melanin present but with the relative degree of expansion or contraction of the pigment masses within the melanophores.

In discussing the goby experiments of Sumner & Doudoroff (1937), I have already pointed out that the more brightly lighted fishes produced on the whole slightly more melanin than the less brightly lighted ones. This was not true, however, of the fishes in white aquaria, in which the amounts were nearly equal, and indeed the relation did not seem to be clearly established in any case. In the much less satisfactory *Gambusia* experiment (Sumner & Doudoroff, 1938*a*), any such difference was even less probable.

It seemed highly desirable to test more precisely the part which was played, if any, by light intensity in influencing the production or elimination of melanophores. This was undertaken in one of the experiments with *Lebistes* (Sumner, 1940). The degrees of illumination received by the aquaria, in the four compartments of the cabinet here employed, were approximately 64, 5·3, 0·85 and 0·24 foot-candles respectively. Special steps were taken to keep the temperature in these compartments approximately equal.

Although the primary object of this experiment was to test the possible effects of light intensity, aquaria of two shades (black and medium grey) were used with each degree of illumination.

When the mean values for fishes from these four compartments were computed, a steady, though slight increase in pigmentation was shown in passing from the lower to the higher illuminations. However, there is little consistency in this respect when the figures for the two backgrounds are considered separately. Moreover, the single differences are of little statistical significance. Nevertheless, the differences are in the same direction as those shown in the earlier experiment by Sumner & Doudoroff (see above). The absence of any obvious effect of light intensity upon xanthophyll formation in this experiment has already been pointed out.

As the limiting case in our discussion of light intensity, we may consider the effect of total darkness upon pigmentation. It is noteworthy that fishes and Amphibia, whether normal or blinded, have usually been found to become pale when kept in the dark, and in a few of these cases the amount of pigment is said to have been involved (Ogneff, 1908; Babák, 1913; Murisier, 1920–1; Odiorne, 1937). Murisier found that the number of melanophores, per unit area of the skin, in young trout which were kept in the dark, was little greater than in specimens which had been kept upon a white background in the light, though the melanophores were less than half as numerous as in specimens kept upon a dark background. Odiorne reports

similar, though less marked, changes in *Fundulus*. This somewhat surprising effect of darkness upon the chromatophores has been offered as a possible explanation of the depigmentation of cave animals (Ogneff, 1908).[1]

## IV. BLINDING

Although the pigmental conditions of blinded fishes can hardly be attributed to *optic* stimuli, they may be fairly credited to the withdrawal of these stimuli, or at least to the withdrawal of impulses proceeding from the retina. But whereas total darkness results, as we have seen, in a process of depigmentation, blinding, in the presence of light, results in a marked increase of pigmentation.

Mayerhofer (1909) reported a great increase in the pigmentation of blinded pike (*Esox*) when kept in the light. The lateral dark bands extended themselves downward until large areas of the normally white ventral area were covered with melanophores. This effect did not ensue when the fishes were kept in the dark. Similar results were reported by Šecérov (1909) for *Nemacheilus*.

Von Frisch (1911) recorded a much less convincing case for the trout. After determining the interesting fact that the removal of one eye in this fish caused a functional darkening of the opposite side of the body, he attempted to determine the relative density of the chromatophores on the dark and pale sides. He counted a slightly greater number of these cells per unit area for the darker side, but this difference was not statistically significant. Babák (1913) reports a great increase in visible pigment in blinded *Amblystoma*, kept in the light. This depended in part upon an increase in the number of chromatophores, following an enormous extension of their contained pigment masses. Fischel (1920) obtained similar results, likewise with *Amblystoma*. He called attention to the formation of an immense syncytium of melanophores, covering the entire body, except for the yellow spots. This increase in the black pigmentation extended to some internal parts. Murisier (1920–1) found that blinded trout became even more heavily pigmented than normal fishes kept upon a black background. Similar results have been reported by Giersberg (1934) for the goldfish and by Odiorne (1937) for *Ameiurus*. I myself have obtained confirmatory results in experiments upon *Lebistes reticulatus* (unpublished), though no counts or measurements were attempted in this case. I also found, in agreement with Fischel, that some of the blinded specimens grew to an exceptionally large size.

Since numerous writers have pointed out that blinded fishes soon become dark when well lighted, we should expect them to acquire heavier pigmentation, in accordance with the accepted principle (see below) that transitory colour changes, if prolonged, lead to quantitative ones.

## V. DIRECT ILLUMINATION WITH COLOURED LIGHT

Various investigators have studied the effects of coloured (more or less monochromatic) light upon fishes. Šecérov (1909) performed such experiments, but his results are open to doubt for reasons stated above. Moreover, von Frisch (1911)

[1] It is uncertain to what extent Ogneff attributed this effect to darkness *per se*, and to what extent to diminished food supply.

failed to confirm some of his findings. The results reported by the latter writer, however interesting, relate only to transitory changes and call for no discussion here.

The experiments of Kurz (1920) are more relevant. He exposed the eggs and larvae of *Pleuronectes* and *Esox* to white light, total darkness, and to light of five different colours. No effects were observed in the case of the pike, after three months' exposure to these conditions. For the flounders, on the other hand, Kurz reported certain effects upon the further development of pigment cells once produced, though not upon their origin. White and short-waved light, he states, further the development both of black and coloured pigments; red, yellow and green retard the development of both. Complete darkness strongly retards the development of black and completely suppresses the development of coloured pigments.

## VI. ILLUMINATION FROM BELOW

The well-known experiments of Cunningham upon flatfishes should be mentioned among the possible effects of optic stimuli, although it appears to have been believed by that author that the increase in pigmentation which he observed was due to the direct action of light upon the skin (Cunningham, 1893, 1895; Cunningham & MacMunn, 1893). Cunningham placed young flatfishes in glass-bottomed aquaria, illuminated from below by mirrors. At the end of many months, some of these specimens developed extensive pigmented areas, containing both black and yellow chromatophores, upon the lower, normally uncoloured side of the body. These abnormally situated chromatophores were said to undergo colour changes like those which may be called forth on the normal upper side of the body.

It is uncertain, however, that this unusual development of pigmentation was due to the direct action of light upon the skin. The not very precise account of the actual visual fields of these fishes leaves it possible to suspect that the effects were due to the optical conditions of the experiment, as has been suggested by Odiorne (1937). The actual nature of the field of vision of an animal, when placed some distance above a mirror, is not very obvious. On first thought, one might assume that the lower portion of this field was a uniform, well-lighted area, optically equivalent to an extended white surface; but a little observation shows that this cannot ordinarily be the case. So far as I have observed, too, a fish's immediate reaction, when suspended over a mirror, is very different from its reaction when placed in a white-bottomed dish. In the former case, far from becoming pale, the animal becomes dark or fairly so. This would suggest that the effects of any particular type of illumination upon "physiological" colour change should be carefully ascertained before we attempt to interpret its effects upon pigment production.[1]

The experiments of Herbst & Ascher (1927) were of a similar nature to Cunningham's and are, I believe, subject to the same comment. These authors placed larvae of *Salamandra maculosa* in small, glass-bottomed aquaria, which were covered on the sides and above with opaque materials. Mirrors reflected light from

[1] See, however, Addendum.

below. The salamanders were kept for from 4 to 7 years (!) under these conditions, controls being kept in black-bottomed containers, lighted from above. While the relative extent of the spotted areas underwent little change, there was a marked increase in the intensity of pigmentation on the animals' ventral surfaces. Not only melanin, but the lipochrome (carotenoid) pigment and guanin were all found to have increased. These writers appear to assume that the effects in question were due to the direct action of light upon the chromatophores, and this, of course, may be the correct interpretation, though it is by no means certain.

## VII. SOME IMPLICATIONS OF THE RESPONSE TO BACKGROUND

Evidence has been offered for the conclusion that in fishes kept for considerable periods on various backgrounds, the amount of melanin (or the number of melanophores) varies inversely as the logarithm of the albedo of these backgrounds. This conclusion is of considerable theoretical interest. The analogy has been pointed out (Sumner & Doudoroff, 1937; Sumner, 1939*a*, 1939*b*) between these pigmental relations of fishes and those phenomena of human sense perception upon which the "Weber-Fechner Law" was based. Even the downward displacement of the melanin value for the "black" fishes (Fig. 3), as compared with its "expected" value, might be regarded as giving additional support to this analogy. As is well known, the Weber-Fechner rule, so far as it relates to visual stimuli, breaks down in the realm of feeble intensities, greater relative increments of stimulus being required in order to bring about perceptible increments of sensation. Whether this deficient (even if actually very high) production of melanin upon a background of very low albedo can be regarded as comparable with the human case is, of course, far from certain. But the coincidence is at least suggestive.

Considerable evidence has also been presented which shows that shade of background is a much more potent factor than intensity of illumination in determining the pigmentary response of the animal. From the viewpoint of a fish or amphibian, as has already been remarked, the difference between incident light and background is a purely topographic one, the upper portions of the visual field rating as "incident light", the lower portions as "background". The albedo of a background, as defined earlier, is the ratio between the light which it receives and the light which it reflects. Response to albedo, therefore, would seem to be determined by the relative degrees of illumination of the upper and lower portions of the retina. This "ratio" theory of an animal's response to the shade of the background, and the correlative assumption of a differentiation of functions in different parts of the retina, have received increasing attention in recent years. Although previous discussions have related wholly to colour changes of the transitory type, we may be certain that the phenomena in question extend to quantitative changes as well.

In reviewing briefly some of the investigations which bear upon the relations indicated, we may distinguish discussions of (1) the "ratio theory", and (2) the functional differentiation of the retina, although it is evident that these two conceptions are closely interconnected. The former appears to have first been clearly stated by Keeble & Gamble (1904) for certain crustaceans. The same formulation

was independently made by myself (1911) as a result of experiments upon flatfishes, though no specific test of the theory was then undertaken. Mast (1916) undertook such an experimental test, likewise with flatfishes. His results were partially confirmatory, though in some respects inconclusive. What seemed to be a critical demonstration of the proposition was made by Sumner & Keys (1929) by the use of an apparatus which made it possible to brighten or darken the background, to some extent independently of the overhead illumination. These findings were confirmed for young catfish by Pearson (1930), by the use of similar methods, though Pearson

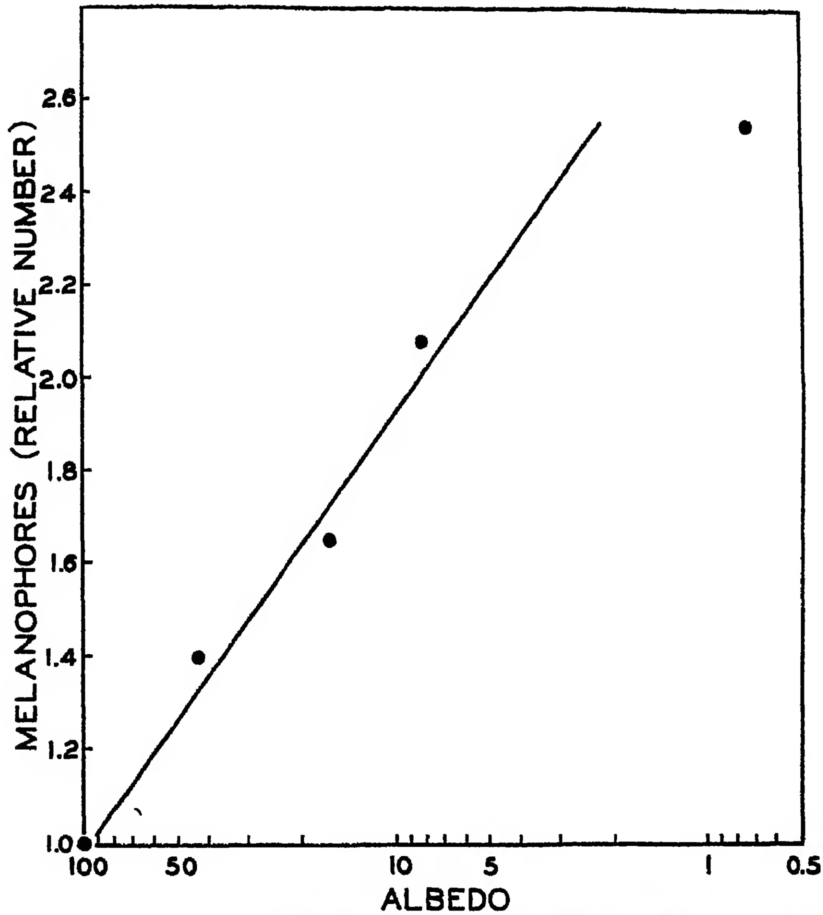


Fig. 3. Same values for melanophores as in Fig. 2, plotted against the logarithms of the albedos.

has insisted upon the limited range within which the rule holds. Brown (1936), using more exact quantitative methods, corroborated the "ratio theory" for the fish *Ericymba buccata*, except for very low light intensities. Butcher (1939) has carried out an intensive series of experiments with *Fundulus* (presumably *F. heteroclitus*), in which varying relations were maintained between the light intensities above and below the fish. Butcher concludes that "The shade assumed by *Funduli*, therefore, depends upon the ratio between the direct and the reflected light entering the eye".

Conclusions relative to a functional differentiation of different portions of the retina have been reached by a number of writers, not all of whom have been concerned with the "ratio" theory of response to background. Suggestions to this effect have been made by Schnurmann (1920), Meyer (1931), Hogben & Slome (1936), and Vilter (1938).

Von Frisch (1911), Parker & Lanchner (1922), Sumner (1933), Butcher & Adelmann (1937) and Butcher (1938) have employed the method of screening the whole or portions of the eye with opaque materials. Sumner, Butcher and Adelmann agree that stimulation of the upper and lower halves of the retina have opposite effects. Other factors remaining constant, the illumination of the upper half leads to a paling of the fish, while illumination of the lower half leads to a darkening. Conversely, darkening the upper half of the retina leads to a darkening of the fish, while darkening of the lower half leads to a paling. Von Frisch (1911) had observed the darkening effect of covering the lower half of the cornea for brief periods (i.e. shading the upper half of the retina), but he believed that covering the upper half of the cornea led to no result.

Several writers have performed the experiment of rotating the eye of a fish in its socket through 180°, thus reversing the normal relations of the retina to the visual field. Sumner (1933) obtained no instructive results from this procedure, owing to the serious (ultimately fatal) effect of the operation upon the fishes. Butcher & Adelman (1937) and Butcher (1938) report success in a number of cases, with results which conform to expectations based upon the results of their other experiments. Butcher accepts the "ratio" theory with some modifications. Vilter (1937, 1938) reports success in rotating the eyes of the axolotl. There ensues, he says, an inversion of the dorso-ventral differentiation of the skin pigmentation, the ventral surface darkening and the dorsal surface paling. Vilter states, however, that such an "inverted" specimen darkens upon a dark bottom, as well as a normal specimen. He offers an explanation based on the respective roles which he attributes to nervous and the hormonal control of the chromatophores.

It may be well to repeat that none of these experiments relative to functional differentiations of the retina have had in view the production of quantitative pigmental changes.

## VIII. THE RELATION BETWEEN "PHYSIOLOGICAL" AND "MORPHOLOGICAL" COLOUR CHANGES

Although we are not concerned here with invertebrates, it is worth noting that Keeble & Gamble (1904) recognized the activity of the crustacean chromatophores in "manufacturing and storing or transforming pigmentary substances", through the mediation of the eyes and nervous system.

Babák noted many years ago (1910, 1912, 1913) that prolonged "expansion" or "contraction" of chromatophores led to quantitative changes in these. With respect to this fact, he offered the following explanation (*trans.*): "We can comprehend all these phenomena consistently if we suppose that the continued expan-

sion of the chromatophores [however brought about] so alters the nutritive condition of the pigment cells that not only an increase of pigment will be brought about in the latter, but also favourable conditions for rapid division, i.e. for chromatophore multiplication, will be produced. On the contrary, the continued contraction of the chromatophores [however brought about] is bound up not only with weaker pigment formation, or even pigment reduction, but also with the checking of the nutritive processes leading to the division of the pigment cells." Babák points out the ecological significance of this identity between conditions which call forth "expansion" of the chromatophores and ones which call forth a permanent increase in the amount of pigment, and conversely with "contraction".

This recognition of the causal relation between the phenomena of transitory and of quantitative colour change has been called by more than one writer "Babák's Law". Stated merely as a causal relation, it is now generally accepted by workers in this field. It appears to be uncertain, however, whether the states of chromatophore "expansion" and "contraction" directly cause the trophic conditions which lead to the multiplication or elimination of these cells, as Babák, Himmer and Giersberg believe, or whether these are merely parallel results of a common (perhaps hormonal) cause, as was admitted as a possibility by Giersberg, and has recently been maintained by Odiorne.

Himmer (1923) set out to test Babák's contention that "morphological" colour changes are a result of prolonged "physiological" colour changes. He performed the interesting experiment of subjecting larval salamanders to dilute solutions of cocaine, a substance which he found to produce a continued state of contraction of the melanin masses, regardless of any visual stimuli. The result was a reduction in the number of melanophores, a condition which was limited, however, to the larval stages. Neither xanthophores nor guanophores were affected by this treatment.

These results would seem to favour the view that the quantitative reduction of melanin is a direct effect of its being concentrated within the cell. But such observations as Himmer's plainly demand confirmation.

Murisier (1920–1) does not appear to be altogether consistent in his statements on this question. At one point, he expresses the belief that "the contraction of the melanophores arrests the elaboration of their pigment and retards their augmentation in number". Later, however, he states: "The inhibition of pigmentogenesis does not result then from the accumulation of pigment at the centre of the cell, since it already manifests itself in the pre-pigmentary element, before the appearance of melanin granules. One arrives thus at the idea that the centripetal migration of the pigment granules and the arrest of pigmentogenesis represent two parallel but secondary effects of the same primordial cause: the arrest of nutrition of the cell which elaborates the melanin." (*trans.*)

Giersberg (1934) also hesitates between the view that "morphological" colour change is the direct result of "physiological", and the view that both are the result of a common hormonal cause. He concludes that neither relation need hold in all cases. As evidence that a continued "contraction" of the melanophore pigment may result in its diminution, he points, among other things, to the outcome of an

experiment in which he injected a young brown goldfish with adrenalin throughout a period of $1\frac{1}{2}$ years. As a result, this animal ultimately lost its yellow and brown pigments. He suggests, furthermore, that the loss of pigment in a fish kept on a white background is due to the secretion of adrenalin into the blood. Such an experiment as the one cited certainly demands repetition. Furthermore, if this result were confirmed, one might still be very doubtful whether loss of pigment following sojourn upon white is due to the same cause.

Odiorne (1936) expresses unequivocally the view that "morphological colour changes (alterations in pigmentation) and physiological colour changes (arising from pigmentary movements) are phenomena resulting from a common cause. The former are not regarded as dependent upon the latter....The neurohumors which are instrumental in bringing about the pigmentary migrations in *Fundulus* also exert trophic influences upon the melanophores."

This last view accords better with our present knowledge of the "neurohumoral" action of pigmentomotor nerves as developed by Parker and others. It accords better also with the fact that numerous chromatophores may appear in a fish undergoing black adaptation, which do not seem to arise from pre-existing chromatophores (Murisier, 1920–1; Sumner & Wells, 1933).

## IX. NERVOUS AND HORMONAL CONTROL OF CHROMATOPHORES

The question of the mechanism by which stimuli originating in the retina are transmitted to the effector organs, the chromatophores, is only incidental to the primary object of this review, though it is probably the aspect of colour-change physiology which is receiving the greatest attention at the present time.[1] An increasing role is being assigned to hormones in linking the receptor with the effector organs. These are partly well-recognized hormones from endocrine glands (pituitary and adrenal), which may be transmitted through the blood stream in response to retinal stimulation, partly a more hypothetical class of "neurohumors", which seem to be generated at the sympathetic nerve terminals and to diffuse through the tissues. Hormonal control has thus far been studied almost wholly in relation to the transitory "physiological" colour changes, though it is likely that quantitative changes result in time from any influence which brings about the transitory ones.

Pouchet (1876) demonstrated experimentally the control of the chromatophores in teleost fishes through the sympathetic nervous system, and Ballowitz (1893) furnished what seems to be conclusive histological evidence of the intimate connexion between these cells and the nerve terminals. Indeed, with certain modifications, the hypothesis of direct nervous control appears to be universally accepted at the present time for the teleost fishes.

The modifications have been in two directions. (1) Numerous experiments have shown that responses to visual stimuli may occur, though tardily, after this intimate connexion between the nerve fibrils and some particular group of chroma-

[1] Rather extensive bibliographies of this phase of the subject have been given by Parker (1930, 1932, 1936, 1938), who, along with a considerable group of collaborators, has been an outstanding leader in this field during the past two decades.

tophores has been abolished. This has led to the supposition that even in "direct" nervous control, the responses are due to hormones liberated at the nerve terminals and carried to the chromatophores by intercellular diffusion (Meyer, 1931; Parker, 1934*a*; Giersberg, 1934; Veil, 1936; and others).

(2) Evidence has been offered, tending to show that even in teleosts a certain amount of influence may be exerted by blood-borne hormones (chiefly those of the pituitary), which are liberated in response to various stimuli (visual among others), and act directly upon the chromatophores, independently of any local nervous control (Parker, 1934*b*; Giersberg, 1934). So far as teleosts are concerned, such a process is generally believed to be of secondary importance, as compared with more direct forms of nervous control. The blood-borne hormone here involved is usually presumed to be a product (or products) of the pituitary. But Giersberg (1934) has suggested that adrenalin may likewise be liberated into the blood stream, in fishes undergoing white adaptation. It is further contended by Giersberg (1930, 1932) that the coloured chromatophores (lipophores) of the minnow, *Phoxinus*, are not innervated at all, but are regulated entirely by the pituitary secretion infundin. Waring (1936) states that "Zondek & Krohn (1932) have shown that in teleosts, injections of posterior lobe pituitary cause an actual increase in the amount of melanin per chromatophore". This is an interesting result and should be confirmed.

An incidental result of possible importance in this connexion was obtained by Sumner & Doudoroff (1938*b*). In the experiments with *Gambusia*, already discussed (p. 357), in which the fishes were kept for considerable periods upon backgrounds of differing albedo (five shades in one experiment, two in another), it was found that the incidence of a fatal disease was far heavier in the black aquaria than in those of lighter shade. In the first experiment, indeed, nearly twice as many fishes died on black as on the other four shades combined. In this experiment, moreover, the death-rate in the dark grey aquaria rated next to that in the black. The figures from the second experiment revealed no probable relation between the incidence of this disease and the intensity of the illumination. As in the case of melanin production, the determining factor was the background. It is, of course, impossible to say whether these differences in morbidity resulted from the changes of pigmentation *per se*, or from physiological processes underlying these changes. It is, however, easier to suppose that physiologically active substances such as hormones could bring about these results than relatively inactive substances such as melanin or xanthophyll.

In elasmobranchs, the evidence for hormonal control seems to be much clearer. Darkening appears to be due to a water-soluble, blood-borne secretion from the hypophysis. Concerning paling, the evidence appears to be contradictory, or perhaps the situation is different in different species. Direct nervous control has apparently been demonstrated in at least one case (Parker & Porter, 1934), while this has not been found in a number of others. Hogben (1936) postulated the existence of a chromatophore-"contacting" secretion in some elasmobranchs, along with the "expanding" secretion from the pituitary. So far as I know, no quantitative pigmental changes have been recorded for fishes of this group.

It is in the Amphibia that the hormonal control of the chromatophores has been longest and most widely recognized. In this group, colour change appears to be chiefly if not wholly controlled by that means. The effect of extirpating the hypophysis upon the visible pigmentation of amphibian larvae was early shown by Smith (1916) and Allen (1917). The former reported an extensive loss of the black pigment.

Hogben concluded, after a summary of existing knowledge in this field (1924), that "the hypothesis of pituitary secretion fluctuating in correspondence with the action of natural stimuli tending to produce colour response is in the existing state of knowledge adequate, at least in adult Amphibia, to interpret all the salient facts"; and again, "even if a nervous mechanism for regulating colour control exists, it plays no significant role in the rhythm of normal colour response in the Amphibia". Paling, according to this view, results from an inhibition of the secretion of the darkening hormone of the pituitary. More recently, Hogben & Slome (1936) have offered evidence for the existence of both darkening ("B") and paling ("W") substances in the secretion of this gland. It may be said that at present the prevailing view is to the effect that direct (i.e. local) nervous control of the chromatophores is lacking in Amphibia. However, this view is not unanimous.

Vilter (1935, 1938) has recently urged that the control of colour change in the axolotl is "neuro-endocrinienne", specifically "sympathico-hypophysaire". The sympathetic control is inhibitive, tending to "contract" the melanophores and depress their growth and multiplication. Some action of a spatially restricted nature is necessary, he urges, to account for the melanophore patterns in the skin (spotting, and the dorso-ventral contrast in pigmentation). These patterns, at the outset, are said to be purely functional appearances, due to the localized "contraction" of the melanophores. Vilter regards this nervous control as actually being hormonal, however, in the sense of its acting through diffusion hormones, as discussed above. Grafting experiments are offered as evidence of this.

In contrast to the sympathetic control, control by the hypophysis is uniform in its incidence throughout the body; it is melanophore-"expanding" and growth-promoting.

Vilter is disposed to extend his belief in the opposing activities of the hypophysis and sympathetic system to all vertebrates. He cites pathological cases of pigment production, following damage to the sympathetic nerves, and goes so far as to assert that "all natural pigmentation is due to the activity of the hypophysis" (*trans.*).

## X. SUMMARY

Fishes and, in lesser degree, Amphibia respond to backgrounds in such a manner that their shade, and to a certain extent their colour, tend to conform to that of the substratum on which they lie, or over which they swim. The integrity of the eyes and of major portions of the nervous system is essential to these phenomena.

The immediate, transitory or "physiological" colour changes are due to the rearrangement of pigment particles already present. When the effective stimuli are

continued for some days or weeks, changes become evident both in the number of chromatophores and in the pigment contents of each (quantitative or "morphological" colour changes).

All three of the types of chromatophores (melanophores, guanophores, lipophores) are affected by these changes. Dark backgrounds favour the production of melanin and inhibit the production of guanin. Pale backgrounds have a reverse effect. In fishes at least, production (or retention) of the yellow pigment xanthophyll is favoured by black backgrounds and retarded by white ones, agreeing thus with melanin. To what extent there is any specific effect of *coloured* backgrounds (*sensu stricto*) upon the quantity of xanthophyll is not clear at present.

Intensity of illumination, above a rather low level, has very little effect upon pigment formation in fishes. There is some evidence, however, of a slight degree of positive correlation between light intensity and melanin formation. Total darkness leads to pigment reduction both in fishes and Amphibia.

Blinding of both eyes, in both of these groups, results in a marked increase of melanin, but only in animals which are kept in the light.

Experiments involving illumination from below are known to have resulted in considerable increases in pigmentation of the ventral surface, both in fishes and Amphibia. It is not certain in these cases whether optic stimuli have been concerned, or whether the effects have been due to direct illumination of the skin.

The response of a fish to its background is primarily a response to albedo, this being defined as the proportion of incident light which is reflected or dispersed from a given surface. On the basis of considerable evidence, a rule has been formulated which has been found to hold approximately, at least for certain fishes. This rule is that, when the animals are subjected to a variety of backgrounds, under uniform illumination, the amount of melanin (or the number of melanophores) produced varies inversely as the logarithm of the albedo of the background. The close analogy between these pigmentary responses of fishes, and the phenomena of sense perception in man for which the "Weber-Fechner Law" was formulated was pointed out.

The question of how a fish recognizes, and responds to, a given albedo, regardless of the absolute degree of illumination present, resolves itself into the question as to how the animal perceives the ratio between the source of light and the light reflected from the bottom and surrounding objects. This last does not seem to be so difficult an achievement when we consider that the ratio in question is ordinarily that between the upper and lower halves of the field of vision, or in other words, between the stimulus received by the lower and upper halves of the retina. Experimental evidence is accumulating showing that these two areas of the retina are functionally differentiated in the required manner.

It was early recognized that those conditions which tend to bring about transitory colour changes are the same ones which, if prolonged, produce quantitative changes. The question has been raised whether the state of chromatophore "expansion" (pigment dispersal) *per se*, promotes pigment production and cell multiplication, and chromatophore "contraction" promotes the reverse processes, or

whether both transitory and quantitative changes are the results of a common (probably hormonal) cause. The weight of present evidence probably favours the latter interpretation.

The relative roles of direct nervous control of the chromatophores and control through hormones is still a subject of controversy, both for fishes and Amphibia. It now seems probable, not only that these two classes of animals differ from one another in important ways, but that the two major groups of fishes, elasmobranchs and teleosts, likewise differ from one another. Even within the group of elasmobranchs, moreover, important differences have been claimed. The whole subject is further complicated by the discovery that "direct" nervous control is itself probably mediated through hormones liberated by the nerve terminals. In general, it is now believed that nervous control (with the reservation just indicated) is the one chiefly involved in the colour changes of teleosts, while control through blood-borne hormones is chiefly involved in the colour changes of both elasmobranchs and Amphibia. But this statement oversimplifies the present state of the problem.

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## ADDENDUM

Since the foregoing review was prepared, Osborn (1940, *Proc. nat. Acad. Sci. Wash.* **26**, 155–61) has reported the results of recent experiments in illuminating flounders from below. This procedure resulted in a considerable development of pigment upon the lower, normally white, surface of the body, both in normal and in blinded fishes. These results were obtained with well-grown specimens (11 to 17 inches). The effects were manifest within a few weeks. The fact that blinded fishes kept on white backgrounds lighted from above also developed "unmistakable amounts of ventral pigment" suggests, as Osborn remarks, that vision is not essential to this process. But I think it likewise suggests that in his experiments with normal fishes the development of ventral pigmentation was not necessarily a direct response of the skin to illumination.

# THE CRITERIA OF PURITY USED IN THE STUDY OF LARGE MOLECULES OF BIOLOGICAL ORIGIN

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(*Received* 22 *February* 1940)

CONTENTS



## I. A DISCUSSION OF THE TERMS USED

Three words in the title need consideration: "purity", "large" and "molecules". The first is the subject that we are dealing with and the other two will simply be defined in a manner useful in the context of this review.

A convenient minimum size for a "large molecule" is the limit reached by existing methods for the synthesis of organic molecules of accurately known structure but not built up by the polymerization of simple substances such as ethylene oxide (Lourenço, 1863) or mixtures of phenol and formaldehyde (Baekeland, 1909). This limit is chosen because synthesis, and a comparison of the properties of the synthetic product with those of the natural product is one of the most reliable tests for the purity of the natural substance. The products of a synthesis are not necessarily pure, but there is a high probability that any impurities present, if they are not breakdown products of the substance concerned, will differ from those present in the naturally occurring material. If, then, the natural product behaves in the same way as the synthetic, it is likely that the behaviour is due to the substance which it was one's intention to synthesize. This point is already fully appreciated by the more prudent workers (e.g. Fildes &

Richardson, 1935), and the recognition of differences between natural and synthetic products has been instrumental in the discovery of many new phenomena.

The molecular weight of a synthetic product can generally be deduced from the method of synthesis, and the physical measurements are used to confirm this value. There is no such *a priori* expectation in the case of a polymer and it is necessary to use the same physical methods both for assessing its homogeneity and for determining its molecular weight. Furthermore, polymers generally consist of a mixture of substances with very similar properties but covering a range of molecular sizes.

The molecular weight limit of partly or totally synthesized organic molecules appears to be 5000 if we exclude those that consist largely of heavy atoms such as tungsten and bromine. Odén (1918), in connexion with his work on humus, prepared several derivatives of raffinose, the hendeca-cerotyl ester of raffinose has a molecular weight of 4669. Of a similar order of magnitude are Fischer & Freudenberg's (1913) hexa(tribenzoyl-galloyl) mannitol (M.W. 2967) and hepta(tribenzoyl-galloyl)-*p*-iodophenyl maltosazone (M.W. 4021), Fourneau *et al.* (1924), in the course of making the trypanocide "309", prepared a series of drugs related to Trypan violet with molecular weights as large as 1626. This method of defining the lower limit to the subject agrees satisfactorily with Staudinger's (1932) distinction between eucolloids (M.W. > 10,000) and hemicolloids (M.W. 1000–10,000).

It is impossible to frame a similar arbitrary definition of the upper limit in molecular weight because of the uncertainty that exists in the distinction between large molecules and particles; the standard definition of a molecule as "the smallest particle showing all the *chemical* properties of the material" merely substitutes for this uncertainty an uncertainty about the differences between chemical and physical properties. Ultimately the distinction between a molecule and a particle (in the usual biological material this would be a fragment from a tissue or bacterium) or association of molecules depends on the stability of the link or links holding the parts together. Staudinger (1935) suggests that the relationship between the particle weight, the concentration of a solution, and its viscosity, is constant for a wide range of particle weights if molecules of the same general character are considered. If therefore the lower members of a series are known to be held together by primary valencies and give the same relationship as the higher members of a series, the latter are also held together by primary valencies and are therefore molecules, whereas if the relationship changes it can be concluded that subsidiary valencies are involved. Although the principle underlying this idea is no doubt sound, it can hardly be applied generally to materials of biological origin since we have not the necessary models of low molecular weight.

The group of substances with which we are concerned in this review lies in the zone between microscopic particles and associations of molecules of known structure, and the word "molecule" will, for convenience, be extended to include any submicroscopic particles that have been studied by chemical or physico-chemical means. In the extreme case of particles visible under the microscope, differences in the appearance or staining properties of the different parts can generally be used as evidence that we are dealing with a mixture of molecules held together by forces

that are as yet inadequately explained. With similar particles it is usual to make the arbitrary distinction on the basis of the ease with which the constituents are separable from one another. The stability of bonds drawn in accordance with the usual chemical conventions may vary greatly with different substituents on the atoms linked together. For example, Bergmann *et al.* (1929, 1930) have found that the ease of hydrolysis of a peptide link is greatly affected by the character of neighbouring substituents and the difference in the stability of the C—C linkage in a paraffin chain and in hexaphenyl ethane is well known. Through the study of the co-ordination compounds and molecular compounds (cf. Bennett & Baker, 1931; Hückel, 1934) a large field of chemistry is being opened up in which, although the law of multiple proportions may hold rigidly, very weak valency forces are involved. These studies offer a useful analogy for that type of loose intermolecular combination which plays such an important role in biochemistry but they do not give us any very reliable means of classifying these substances. During the past decade there has been no change in the position clearly described by Sørensen (1930): "Hence a rational classification of chemical combinations based on the type and strength of the valencies effective in the combinations is hardly producible. It is easy enough to set up typical representatives of each group of the classification, but at the same time it must be conceded that all kinds of transition links exist between the groups. These difficulties also make themselves felt in the choice of classification principles and of the nomenclature capable of suitably characterising the behaviour of high molecular substances."

Willstätter & Rohdewald (1934) use the term "symplex" for an association of substances one or both of which has large molecular weight, it is also used by Przyłecki (1939) to cover a range of natural and artificial products. These authors have made no attempt to define the nature or stability of the linkage which holds the constituents of a substance in the symplex category together. It is therefore doubtful whether the introduction of an undefined term intermediate between molecule and particle is useful.

Meyer & Mark (1928) and Ostwald (1929) have opposed the extension of the original meaning of the words "molecule" and "molecular weight" to cover this group of substances and they suggest the use of Nageli's word "micelle" instead. Sørensen, however, although he excludes the lipoproteins from the category on the ground of their lability (1930), finds "molecule" a satisfactory descriptive term for the other proteins that he has studied and it has been used by most workers on the water soluble proteins during the last few years. Ostwald's objection was due, not to the weakness of the linkages, but to the inhomogeneity of the materials with which he was concerned, whereas Sørensen and Willstätter would not use the word in some cases where physical measurements suggest that the preparation consists of particles of the same weight.

If we say that a particle has a molecular weight of 50,000,000 we have prejudged the question of whether it should be called a molecule or not; on the other hand, the periphrasis "having a weight equal to that of a molecule of molecular weight 50,000,000" is clumsy. There seem to be three ways of avoiding this difficulty:

(1) To speak, as Bawden (1939) has done, of particles weighing 50,000,000 times as much as a hydrogen atom.

(2) To measure all these weights in grams or micrograms.

(3) To use a symbol for one-sixteenth of the weight of an oxygen atom and *then to refer to particle weights in terms of this unit.*

The first course is objectionable in theory only, for the difference between the atomic weight of hydrogen (1·008) and 1·0 on the atomic weight scale (oxygen = 16) is insignificant in most measurements on large molecules. There is little to be said for the second course which leads to unwieldy numbers, and to difficulties in any statement involving molecular weights of the smaller proteins as well as these large particle weights. The third course is an extension of molecular weight nomenclature that substitutes an absolute unit of $1 \cdot 65 \times 10^{-24}$ g. for the ratios normally used. The symbol "h." might usefully stand for this quantity, and, if we follow the usual rules of symbolism (cf. Hill, 1935), Mh. would be 1,000,000 times this quantity or $1 \cdot 65 \times 10^{-18}$ g. In the example already used the particle would therefore have a weight of 50 Mh. The symbol Mh. would be that normally employed, for with smaller particles there is more rarely any objection to speaking of molecular weight; confusion will not therefore arise between the new symbol and Planck's constant, and the advantages of a symbol that suggests a relationship with the hydrogen atom can be fully exploited.

From the discussion of the significance attached by different chemists to the word "molecule" it is clear that no hard and fast lines can be drawn. It is unfortunate, therefore, that in some biological writing effort should be expended on a discussion of whether or not structures such as viruses or genes are molecules (e.g. Stanley, 1938 *a*, *b*; Gulick, 1939). If the issue is in doubt the word should be avoided, for the properties and potentialities of a virus are in no way circumscribed by the demonstration that it is often possible to study it with the techniques used in the study of admitted molecules and this is all that the work on the purification of viruses has done. The word "molecule" tends, and is often intended, to imply a larger body of exact information about a structure than we do in fact possess. Like the word "crystal" it very often conveys a false impression even although a direct misstatement has not been made; in other words it "lends an air of verisimilitude to an otherwise bald and unconvincing narrative".

## II. FOUR IMPLICATIONS OF THE WORD "PURE"

In many branches of organic and inorganic chemistry the behaviour of a substance on distillation or on crystallization and recrystallization, the constancy of its analytical figures, the perfection of the agreement between these figures and those calculated for some simple formula and a comparison between the properties of preparations made by different methods, are criteria of purity which satisfy all but the most pedantic. There are also, however, groups of substances, e.g. derivatives of the elements of the rare earth group and some lipoids, where these criteria are either inapplicable or no longer reliable. Proteins, polysaccharides and other

molecules large enough to be included within the limits defined in the preceding section, must be compared with this second group.

At a certain degree of complexity the word "pure" takes on connotations different from those that it has in chemical usage, and there is a danger that this change of meaning may cause confusion. There is, for example, no chemical significance in the phrases "a pure culture of *Br. melitensis*" or "a pure strain of mice", although each has a definable bacteriological or genetical meaning. The implications of the word when applied to a preparation that has a measurable activity, e.g. an enzyme, an antigen or a virus, can be considered under four main headings:

(1) The preparation may contain particles identical in size and in chemical composition and each carrying the full unmodified activity of the starting material.

(2) The preparation may be chemically and physically homogeneous but all the particles may have been altered by manipulation so that they are of reduced or enhanced activity or they may have lost some aspects of their activity. The preparation, although all the constituent particles are of identical size and composition, is not therefore pure in the first sense because it is a different substance from that present at the beginning.

(3) With particles built up by the repetition of a subunit the particles may be of similar composition and they may all be active but they may cover a range of sizes.

(4) The preparation may consist of a mixture of chemically and physically distinguishable substances each of which carries the activity described.

### (1) *The ideal case*

In general, it is the first meaning that is understood when the word "pure" is used, and if it is claimed that a substance is pure in this sense the only difficulty that arises is that of getting the necessary evidence. In theory attention should also be paid to the isotopic constitution of a substance, for differences in the physiological effects of isotopes have been noticed. Brewer (1937) and Lasnitzki & Brewer (1938), for example, have found that the proportion of the potassium isotope with atomic weight 41 is greater in bone marrow and smaller in the Jensen sarcoma than in other tissues or in mineral deposits. There is a tendency to minimize the possible importance of isotopes because the percentage of the heavy isotope in the four commonest biological elements, hydrogen, carbon, nitrogen and oxygen, is small (0·02, 0·7, 0·38, 0·2 % respectively); however, other elements of known biological importance have their isotopes more evenly balanced, e.g. chlorine, copper and zinc (24·6, 32 and in the case of zinc 27·3 and 17·4 % respectively). With these elements the difference between the isotopes is smaller because of the greater atomic weight, but it is well to bear in mind that compounds containing such elements are mixtures of similar quantities of two or more substances. At the present stage in the development of biochemistry this refinement in our conceptions of purity is probably unnecessary.

### (2) *Substances modified during isolation*

The difficulties that arise in considering substances that have been modified in the sense of heading (2) are due primarily to the absence of a satisfactory standard for the initial activity. With substances that occur in nature as solutions, such as secretions or sera, or with substances that are exclusively studied as solutions, standards can be set up. It is then possible to compare the crude with the purified material and to consider whether any differences that are noticed are due to changes in the molecule or to other constituents in the crude mixture. The soluble specific substance of type I pneumococcus is a case in point, for several workers (e.g. Felton, 1932; Wadsworth & Brown, 1933) have found that products isolated by the technique of Heidelberger *et al.* (1925) differed from crude bacterial extracts in precipitability and immunizing ability. Avery & Goebel (1933) showed that the earlier product was a deacetylated derivative of the carbohydrate present in these crude extracts and that, if deacetylation is avoided, there is a close correspondence between the immunological properties of crude and purified material.

Chlorophyll and haemoglobin can be studied in considerable detail while still inside the chloroplast or corpuscle. The differences in solubility and absorption spectrum between chlorophyll in the chloroplast and after isolation are due apparently to its presence as a complex in the chloroplast, and Smith (1938) has prepared extracts which resemble the chloroplast material more closely than chlorophyll isolated by the usual procedures. The state of haemoglobin in the corpuscle may be similar. From a study of the oxygen dissociation curve of corpuscles and of purified haemoglobin Altschul *et al.* (1939) conclude: "In the erythrocytes haemoglobin is either in a different form from that obtained by our method of preparation or it is associated with some other substance. From the biological point of view haemoglobin, as it exists in the cell, is of greater significance, while from the chemical view-point, this 'purified' form may very probably be of greater interest." The truth of this statement will depend on whether we look on haemoglobin as a substance defined by a certain method of purification or as the oxygen-transporting material in the corpuscle, for in the latter case, if it is combined in the corpuscle, it is no longer haemoglobin when free. Adams (1938) finds that corpuscles lack an absorption band at 410 m$\mu$ which is shown by haemoglobin or by laked blood; this band disappeared from haemoglobin solutions when they were incubated with a stroma protein preparation. Adams suggests that in the corpuscle there is a complex of this type. The effect on the absorption of light would seem to be noticeable in only a narrow region of the spectrum, for Drabkin & Singer (1939) find no difference in the 500–630 m$\mu$ region between the spectra of isolated haemoglobin and of corpuscles after correction for the effects of scattering.

The various preparations of tobacco mosaic virus that have been described illustrate changes of a somewhat different type that may be undergone by a substance during the course of isolation. Preparations made before 1935 were either not described chemically or else they consisted largely of material having no connexion with the virus. Stanley (1936, 1937) described preparations which,

although they could properly be called proteins, consisted largely of either extraneous protein or protein derived by the breakdown of the virus that had been present in the sap. It is these preparations that have been described as crystalline. The preparations made by Bawden *et al.* (1936, 1937 *a*) were also proteins, but their description of the virus differed so completely from that published by Stanley that only the infectivity suggested that the same substance was being handled. These differences covered physical properties, chemical composition and serological precipitation. Bawden & Pirie (1937 *a*) pointed out that when the purification was carried out by the methods so far described, methods that have been in use in protein chemistry for fifty years, the virus suffered an irreversible change. This change leads to lower infectivity and filterability but not apparently to changes in chemical composition or serological behaviour; all preparations which appear fibrous or para-crystalline under the microscope have been made by treatments, e.g. precipitation with acid or ammonium sulphate, which bring about this change. There is reason to think that this change or aggregation (Bawden & Pirie, 1938 *a*) may occur naturally in old or necrotic plants, but it seems reasonable to look on the most active naturally occurring material as our standard for tobacco mosaic virus. More recently preparations have been made by high-speed centrifugation at low temperatures in which this change had been avoided or at any rate minimized (Loring *et al.*, 1938). , Repeated centrifugation is necessary to get colourless preparations of constant analytical composition, and on repeated centrifugation there is apparently a fall in infectivity and filterability although not to the level reached in preparations rigorously purified by ammonium sulphate precipitation. It has not as yet been shown that preparations that are in the fully infective state are free from other plant constituents, nor has it been shown that they are homogeneous by other criteria. It would appear therefore that preparations that contain tobacco mosaic virus in its most infective state have not yet been shown to have sufficient chemical and physical homogeneity to make the discussion of their purity profitable, whereas the apparently homogeneous preparations can no longer be looked on as unmodified virus. The work of Bawden & Pirie (1938 *a*) and Loring (1938) suggests that somewhat similar changes take place during the isolation of potato virus "*X*", but there is reason to associate these changes with the removal of a polysaccharide.

### (3) *Isochemical substances*

Brønsted (1931) described substances as "isochemical" when they did not differ essentially in chemical properties but only in molecular or particle weight, and he has discussed the effects of these differences in weight on the partition of the substances between different phases. Especially in cases where association and dissociation is reversible or where the starting material is likewise heterogeneous, such a product could legitimately be called pure. Substances that have been rendered soluble by the mechanical fragmentation of a coarser material would be expected to be isochemical, and Beckmann & Landis (1939 *a*, *b*) have studied in the ultracentrifuge amylose preparations made by the dry grinding of corn starch. Isochemical mixtures are generally encountered either as the result of such mechanical

treatment or among the products of polymerization reactions. The chemical properties of all the fractions of a mixture made in these ways need not be identical but only similar, and as an example of this the well-known effect of previous treatments such as milling on the reactivity of cellulose preparations towards iodine or acetylating agents, may be cited.

Sabin's (1939) picture of the origin of normal and antibody globulins is interesting in this connexion. She suggests that the outer surface of the macrophages is shed in a manner correlated with antibody formation and suggests that antibodies are made by the dispersal of these fragments in the serum. It might have been expected that soluble proteins arising in this way would be inhomogeneous with respect to molecular weight as was observed with the native antigen of *Brucella melitensis* for which Miles & Pirie (1939 *c*) suggested a somewhat similar origin on the surface of the bacterial cell. Many centrifugal analyses (e.g. Kabat, 1939) have, however, shown that antibodies, unless derived from animals that have been immunized over a long period, are relatively homogeneous. In terms of these hypotheses therefore we must picture the macrophage surface as subject to a much more uniform dispersal than the *Brucella* surface, i.e. the one breaks up like a tiled and the other like a cement floor. The two products have this in common, that each shows anisotropy of flow and Kabat (1939) finds that horse antibody particles are more anisodimensional than those of any other protein for which figures have been published (cf. table in Svedberg, 1939).

Reversible associations and dissociations have been found by many workers for many substances, e.g. Tiselius & Horsfall (1939) studied the effect of *p*H changes on the haemocyanins, Hand (1939) the effect of dilution on urease, and Miles & Pirie (1939 *b*) the effect of sodium dodecyl sulphate and ammonium sulphate on the soluble specific substance of *Brucella melitensis*. These treatments changed the molecular or particle weights by a factor of 8 or more, whereas isochemical inhomogeneity in Brønsted's sense does not cover a range as great as this. Small variations in the molecular weight of a substance are more likely to occur among molecules that are built up from a repeat unit than among the proteins. The impossibility of dividing a protein into two or more identical pieces, i.e. the absence of any repeating structure, is an important feature of the Bergmann & Niemann (1937) hypothesis of protein composition. Furthermore, since the amino-acids are looked upon as forming a set of interpenetrating sequences in which each occurs a number of times expressible as $2^n \times 3^m$ (where $n$ and $m$ are integers), there are not many ways in which pieces could be cut from or added to the structure. It will, however, be generally conceded that this hypothesis is simply a valuable approximation to a picture of protein structure and that, since it depends on the precision of amino-acid estimations, its uncertainty will become great when the molecular weight of the protein exceeds about 200,000. Inhomogeneous proteins are of course well known, but either as a result of pathological conditions where it is reasonable to assume that there has been a breakdown of normal protein (McFarlane, 1935) or as a result of the treatments the protein has received during isolation. Until one of these inhomogeneous products is fractionated and the extent of the chemical

difference between components of different weight is determined it is impossible profitably to discuss whether they fall within the isochemical zone or not.

Since the proteins that have been studied are built up from a number of different amino-acids that is generally between one-tenth and one-sixtieth of the total number of amino-acid residues present, it will only rarely be possible to have slight changes in the molecular weight without at the same time having significant changes in the proportions of the different amino-acids. Changes of this type are referred to as "protein plasticity", and evidence that they occur as a result of changes in the diet and environment of the animal that is making the protein, has been published by Roche (1935), by Roche & Roche (1936) and by others. Clearly the formation of antibodies shows that some cells, as a result of an altered environment, can make proteins with different properties, but it has not been conclusively shown that other protein-synthesizing cells share this property. It still seems to be possible to explain the phenomena covered by the term "protein plasticity" by variations in the ratio in which two or more proteins are made, by variations in *p*H causing different degrees of dissociation, as in the experiments of Tiselius & Horsfall (1939) on the haemocyanins, or by minor variations in amino-acid content caused by chemical or enzymic action on the unhydrolysed protein (cf. Schmidt *et al.* 1940) rather than by variations in the original constitution of an individual protein.

### (4) *Substances that share a name*

When substances are chemically and physically distinguishable but share a common property any confusion that may arise through the early use of the same name to cover all of them is purely verbal and is easily resolved by retaining the old name as the name for the group and giving to each individual its own name. Two examples of mixtures that have recently been separated in this way may be given: heparin, which Jorpes & Bergstrom (1939) claim is a series of substances with varying sulphate content, and pepsin, which Desreux & Herriott (1939) find consists of several components even when it is derived from one animal species.

## III. ASSESSMENT OF PURITY BY CHEMICAL ANALYSIS

Elementary analysis is of great value in suggesting to which group of substances a product belongs and in assigning upper limits to the extent of possible contamination by specified substances. The different members of a group, e.g. the polysaccharides, have as a rule such similar chemical composition that elementary analysis can give no further information about the state or constituents of a preparation. Similarly, when it is known that a preparation consists largely of protein it is clear that the carbon, hydrogen and nitrogen content has little relevance in the discussion of its purity, for all proteins give similar figures and it is more satisfactory to demonstrate the presence of non-protein material by direct estimation rather than by inference from low nitrogen contents. At best these analyses could be used to show the regularity with which the same product can be obtained; when used in this way, however, the figures should not vary from paper to paper as much as

those of some supposedly pure plant viruses. Analyses for elements which occur in smaller quantities, e.g. phosphorus, sulphur and metals, are of more value, and so are estimations of groups such as sugars or amino-acids. It is unfortunate that the latter often involve the use of methods with low precision and specificity. The principle that a protein molecule must contain one residue of each of its constituent amino-acids has often been used to establish the minimum molecular weight, but, if some other estimate of the molecular weight is available, it is of greater use as a criterion of purity. For example, the assumption that the silk fibroin in which Vickery & Block (1931) found 0·07 % of histidine was a mixture is as probable as Bergmann & Niemann's assumption (1938) that it was pure and had a molecular weight of 217,700. Accurate metal estimations give valuable evidence on the uniformity of successive fractions of a product, and they suggest that several proteins, which had appeared to be homogeneous, are in fact mixtures. Thus haemoglobin, although it is known not in general to form mixed crystals with the haemoglobins of other species, seems to carry a copper-containing protein with it after repeated crystallization, for Elvehjem *et al.* (1929) have found 0·0013–0·003 % of copper. This would necessitate a molecular weight of over a million if the copper is not an impurity. Furthermore, if concanavalin A contains 0·023 % of manganese, as Sumner *et al.* (1938 *b*) find, its molecular weight would have to be some multiple of 240,000 rather than 96,000 as they found centrifugally.

It is clear therefore that analysis may shed much light on the chemical homogeneity of products that seem homogeneous by physical measurements, for it gives an entirely independent method for the determination of an integral fraction of the molecular weight. The physical determination of the molecular weight need only give a rough or, as in the case of osmotic pressure measurements, an average value for the substances present.

## IV. CRYSTALLINITY

Of the various criteria of purity that are used and accepted in the study of large molecules crystallinity is the most misleading. Fortunately it is now becoming usual to speak of the uncertainty of crystallization as an index of protein purity, and the point, appreciated by some at the end of last century (e.g. Wichmann, 1899), has been mentioned by several reviewers in recent numbers of the *Annual Review of Biochemistry*. These same reviewers, however, betray by turns of phrasing the unmerited importance that they attach to the crystallinity of a preparation. No one can deny that crystalline preparations are more pleasant to handle, more beautiful, and in general contain fewer constituents than amorphous preparations, but it would be unwise to praise them more highly than that, for recent experience has shown that they are probably, rather than just possibly, contaminated. Crystalline preparations of serum albumin (Hewitt, 1936; Luetscher, 1939), trypsin (Dubos, 1937), pepsin (Desreux & Herriott, 1939; Agren & Hammarsten, 1937 *b*), urease (Sumner *et al.* 1938 *a*) and catalase (Agner, 1939) have all contained other proteins. In some cases these, having been recognized, have been removed, but the important fact remains that preparations that seem homogeneous under the microscope have

contained as many as five components, some of which have even differed widely from the basic material in sedimentation constant, and that this contamination has had to be recognized by some technique other than recrystallization. Tomato bushy stunt virus preparations as active as any that have been prepared will crystallize when mixed with any proportion of the non-infective nucleoprotein (Bawden & Pirie, 1938 *b*) that can be made from it. Similarly, the microscopical appearance of the paracrystals of tobacco mosaic virus is unaffected by the presence of normal plant proteins, of other viruses of the tobacco mosaic type and of large quantities of tobacco mosaic virus breakdown products (Bawden & Pirie, 1937 *a*). In any field of chemistry crystals may be contaminated with a fraction of 1% of other material, but the protein crystals mentioned above may contain similar quantities of all of their components. When an author describes the preparation of a crystalline protein from a tissue he may well be understating the case if he only claims to have crystallized *one* enzyme; the preparation should not be referred to as crystals of the enzyme that is being looked for unless it has been shown to be free from most of the other enzymes that are known to occur in the tissue used.

Properly speaking, the characteristic property of a crystal is the regular ordering of the constituent molecules and this is recognized primarily by the rational indices of its faces or by an X-ray study of the reflexion planes inside the crystal. When crystallinity is adduced as evidence of the purity of a substance all that has been observed in many cases is the presence of flattened surfaces. Until more is known about the forces that tend to produce flattened surfaces on a particle, it is unwise to attach too much importance to these facets. One does not, for example, look on the starch grains from *Zostera marina* or *Gladiolus byzantinus* as purer than the more usual amorphous grains because it is generally believed that the regular facets, which Reichert (1913, 1919) photographed so beautifully and which sometimes give the grains a regular dodecahedral appearance, are due to external pressure during the building up of the grain. Langmuir (1938) looks on a protein crystal as a more rigid form of a tactoid, the latter being a liquid droplet or coazervate which has taken on some degree of orientation, but neither he nor Razumovski (1939), who likewise has considered the crystallization of large molecules, gives any treatment of the mechanism of facet formation. It is well known to protein chemists and the point is illustrated in the photographs of haemocuprein published by Mann & Keilin (1938), that a preliminary to crystallization is often the separation of rounded particles with the shape of the liquid droplets that Zocher & Jacobsohn (1929) and Freundlich *et al.* (1933) have called tactoids. Either on standing or on recrystallization these tactoids may develop facets. The importance of facets has been reiterated because experience shows that the readiness with which a conventional scientist becomes convinced of the purity of a protein preparation depends more on the beauty of its crystalline appearance than on anything else.

A crystalline preparation may be contaminated in three ways: by amorphous material; by crystals of other material; by being a mixed crystal or, to use van't Hoff's preferable nomenclature, a solid solution. The first two are of less interest and

present fewer obstacles to further purification than the last. Solid solutions have received more attention from mineralogists and metallurgists than from organic chemists, and it is probably primarily for this reason that they are not recognized more often in protein chemistry. Jost (1937) has given a somewhat theoretical account of the properties of solid solutions, and the subject was reviewed in a lively and personal manner by Bruni (1925). It is reasonable to suppose that the individual members of the group of crystallizable proteins contain in general fewer constituents than the individual members of the group of non-crystallizable proteins, and that the number of constituents is reduced by repeated crystallization. It must, however, be emphasized that repeated crystallization often causes protein denaturation or modification and that nothing will be gained by recrystallization unless it is certain that unwanted constituents are being removed in the mother liquor. Ten recrystallizations have no bearing on a protein's purity if nothing has been removed by the last eight of them.

Certain rules can be formulated which relate the sizes and charges on ions to their tendency to form mixed crystals in minerals. As Goldschmidt has phrased it (1937): "the question arises as to the order of preference in which the different ions, being of suitable size, will enter into a crystal lattice: not all guests are equally welcome in a lattice, and the crystal in many cases makes a choice between them." No principles similar to those of Mitscherlich in the inorganic field have yet been established governing the extension of "hospitality" by one protein crystal to another protein. Bonot (1937) concludes that for syncrystallization it is necessary to have identity of the thermo-elastic properties of proteins, but he presents no evidence that any proteins differ widely in this respect. In view of the readiness with which some substances of similar chemical constitution form solid solutions with one another (cf. the work of Piper *et al.* (1931) on long-chain hydrocarbons and alcohols) the absence of mixed crystals in some groups of proteins is perhaps more surprising than their presence in others. The dissimilarity between the crystals of haemoglobin from different species, emphasized by Reichert & Brown (1909), is a well-known example of this phenomenon.

It is difficult now to understand the prejudice which, ten years ago, existed against the idea of crystalline enzymes or viruses, for it was then generally admitted not only that it was perfectly normal for proteins to take up the crystalline state but also that it was probable that enzymes either were proteins, or contained them. The possibility of crystallizing enzyme preparations is now so generally accepted that there is a tendency to over-rate the purity of the early crystalline preparations. As an example urease may be cited, for Sumner's (1926) and Sumner & Hand's (1928) claim that it would crystallize did much to stimulate interest in the isolation of enzymes and, as a natural consequence, of viruses. It is now known that the crystalline preparations that have so far been prepared contain several components (Sumner *et al.* 1938 *a*). It is probable that one of these components is actually urease and that it also would crystallize if separated from the others, but the distinction between the early crystalline preparations *containing* urease and a crystalline preparation *of* urease should be maintained.

## V. EVIDENCE FROM ULTRACENTRIFUGATION

The fact that a preparation gives a single sharp boundary in the ultracentrifuge is a point of great importance, but four considerations must be borne in mind before it is used in the assessment of purity. In the first place a glance at the photographs published by different workers shows that they attach widely different meanings to the word sharp. This is especially true when the light absorption method rather than a refractive index method of measurement is used (for a description of the different methods of measurement see Svedberg & Pedersen (1940) and Philpot (1938)). Even when the boundary is sharp this may not mean uniformity of particle size if the particles are greatly elongated, as in the case of the methyl celluloses and some plant virus preparations, for, as Signer & Tavel (1938) have shown, particles that differ in length may not differ appreciably in sedimentation constant. Furthermore, a single boundary will naturally be given by a preparation that contains an amount of another component too small to be detectable by the method of measurement used. If the contaminant has the refractive index and intensity of absorption characteristic of proteins this amount may be as large as 5 %.

In the second place it is to be expected in theory and it is observed in practice (e.g. Gralén, 1939; Kabat, 1939) that the rate of sedimentation of a protein will increase as the solution is diluted. This will tend to produce a spurious sharpening of the boundary because the slowest moving particles will lag behind and will therefore tend to travel in the most dilute solution. This is an effect that will be most noticeable with large molecules, for the spreading of the boundary by diffusion is then slight. A spurious sharpness will also be introduced if the protein is centrifuged in the absence of electrolytes of low molecular weight.

The third consideration has a different form according to whether the position of the boundary is ascertained by measuring variations in the refractive index along the fluid column or by measuring its opacity to visible or ultra-violet light. In the first case very inhomogeneous contaminants will be detected with difficulty, in the second case transparent contaminants such as polysaccharides will not be detected at all, and in both cases contaminants of small molecular weight may be missed. Philpot (1939) has corrected for the first of these possibilities of error by comparing the refractive index increment observed over the whole tube with that which would be expected if all the material in the solution had the same sedimentation constant. Some information about the contamination of a preparation with ultra-violet light-absorbing material can be got from a comparison of the opacity of the fluid from which the main component has been centrifuged with that of the air bubble at the inside end of the cell or with that of a cell full of buffer solution (Wyckoff, 1938).

The fourth difficulty in the interpretation of ultracentrifugal evidence of homogeneity is in many respects the most serious. Svedberg has pointed out in many papers that there is a tendency for proteins to have molecular weights that are multiples of 17,600, and this generalization has been restated by Bergmann & Niemann (1937) in the form that a protein molecule contains multiples of 144

amino-acid residues. These generalizations have not met with universal acceptance, but it is at least clear from the tables giving the molecular weights of all the proteins for which reasonably accurate molecular weight measurements have been made (e.g. Svedberg, 1939) that there is a grouping round the values that are demanded by the hypotheses. This regularity robs centrifugal homogeneity of some of its significance as an index of protein purity, for it means that a large number of proteins in an extract may be covered by a much smaller number of molecular weights. If, therefore, a protein that has been isolated from a tissue appears to be centrifugally homogeneous with a sedimentation constant corresponding to the common 35,200 or 70,400 molecular weight groups, it becomes reasonable to wonder whether it may not be a mixture of several different proteins with nearly the same molecular weights. It is well known that proteins exist, both naturally and as a result of changes that have taken place during manipulation, with intermediate molecular weights. The regularity is not therefore a necessity and, if the hypotheses referred to above should turn out to be justified, it is at least as likely that the tendency of amino-acids to appear in groups of a gross is due to a uniformity in the enzymes that are concerned in the making of proteins, as that the tendency is due to some physical requirement for stability.

Similar regularities have not been recognized among the sedimentation constants of polysaccharides and related substances, but the number of observations that have been made in this field, e.g. Svedberg & Gralén on plant gums (1938), Siebert *et al.* (1938) on tuberculin polysaccharide, Beckmann & Landis (1939 *a*, *b*) on amylose, and Philpot (1939) on the specific substance from *Brucella melitensis*, is too small for this absence of regularity to have any significance.

## VI. EVIDENCE FROM ELECTROPHORETIC MEASUREMENTS

Electrophoretic measurements in Tiselius's (1937, 1938) elegant apparatus will detect differences between proteins, e.g. the serum globulins and the haemoglobins, of similar molecular weight, and these measurements offer one of the most reliable criteria that can be applied. Longsworth and MacInnes (1939, 1940) have considered in detail some anomalies in electrophoretic measurements on proteins that have a mechanical or physico-chemical origin. There are however other anomalies due to superficial resemblances between individual proteins. Landsteiner *et al.* (1938) have, however, found that the egg albumins from closely related birds, e.g. chicken, guinea-hen and turkey, have such similar mobilities that, although they are serologically distinguishable they are not electrophoretically separable. As these authors also point out, it is to be expected that proteins of similar type should have similar mobilities if we accept Block's (1934) generalization that proteins of a similar type contain certain amino-acids, especially the basic amino-acids, in the same proportions. Mobility in an electric field, unlike serological specificity, crystalline form and perhaps also solubility, presumably depends on the *number* of acid and basic groups in a protein more than on their spatial distribution on its surface. Tiselius & Kabat (1939) have found that there is a

characteristic mobility for the antibodies that an animal makes, although the work of Moore *et al.* (1939) suggests that this mobility is not necessarily constant in all individuals of the species at all times. It is, however, well known that an animal can contain antibodies to different antigens simultaneously, and Landsteiner & van der Scheer (1936, 1938) have shown that the antibody complex made in response to a single antigen may be a mixture of several serologically distinguishable antibodies. It is clear therefore that there exist between proteins serological differences that are not at present perceptible by electrophoretic measurements.

## VII. EVIDENCE FROM THE PARTITION OF THE SUBSTANCE BETWEEN DIFFERENT PHASES

The possibility that a molecule of the type that we are discussing may be volatile need not be taken very seriously, for claims like that of Olsen & Yasaki (1923) that it is possible to distil a bacteriophage have not found general acceptance. The only equilibria therefore that come up for consideration are those between solids, between liquids and between solids and liquids, and of these the first need not be discussed on account of the slowness with which it would be attained.

Most of the methods used in the fractionation of mixtures containing proteins and polysaccharides, e.g. precipitation with salts, acids, solvents or, more recently, co-ordination compounds (Michael, 1939), involve equilibria between solids and liquids, and it is on the judicious control of conditions such as temperature and the concentration of the reagents that the specificity of these methods depend. When the fractionation has been completed similar agents can be used to assess the purity of the product. Cohn (1921) has pointed out that the solubility of a protein under defined conditions should be constant and independent of the amount of protein present in the solid phase. This was found to be true of carefully purified samples of serum globulin equilibrated with successive quantities of water. Similar methods have been used by many other workers both as indices of purity and as a means of finding whether proteins of different origin are identical or not. For example, Landsteiner & Heidelberger (1923) found that oxyhaemoglobins from the horse and donkey were not appreciably soluble in solutions saturated with the other, and that they are hardly distinguishable serologically although they are distinguishable crystallographically (Reichert & Brown, 1909; Loeb, 1917). On the other hand, Northrop (1933) found that pepsins from the ox and pig dissolved independently. Steinhardt (1939), using a technique similar to that of Cohn (1921), found that pepsin did not apparently obey the Phase Rule, but the interpretation of his results is rendered difficult by Desreux & Herriott's (1939) demonstration that, even when derived from one animal species, pepsin is not a single substance and that the individual components do obey the Phase Rule. Northrop & Kunitz (1930) have published a theoretical treatment of the solubility curves that are to be expected with solid phases that are mixtures, solid solutions and pure substances, and they (Kunitz & Northrop, 1938) have used these relationships in a consideration of the purity of chymotrypsinogen and chymotrypsin. This treatment was extended by

Kunitz (1938), who has studied in detail the conditions under which chymotrypsin and its proteolytically active hydrolysis products form solid solutions or crystal mixtures; the formation of solid solutions here is interesting, for the molecular weights of the constituents range from 40,000 to 27,000.

Northrop & Kunitz, in the papers quoted, have pointed out the possibilities of error which exist when this criterion of purity is used, and it is clear that, although valuable information about a product can be got from solubility measurements, the absence of valid rules governing the formation of solid solutions among proteins makes the method fallible; similar points have been made by Steinhardt (1938) in a discussion of proteins in general and pepsin in particular. As has already been stated, the processes used in protein purification often consist in the separation of the crude material into fractions with similar solubilities in salt solutions. It is essential therefore that solubility measurements, if they are used as evidence of purity rather than as evidence that a certain type of fractionation has been carried to its useful limit, should be made with different salt solutions and at different *p*H values from those used in the fractionation.

The use of a system of two immiscible liquids in fractionation has great advantages, both from a theoretical point of view and practically, because of the convenience of handling liquids and the rapidity with which repeated fractionations can be carried out. These advantages have been thoroughly exploited, both for the separation of small molecules (e.g. Cornish *et al.* 1934; Synge, 1939) and as criteria of purity with liquids (Jones, 1923, 1929). The technique is more difficult to apply when the molecules that are being separated from one another are large, for, as Brønsted has clearly shown (1931, 1938), they tend to partition very unevenly and to occur almost exclusively either in one phase or the other. Laland & Klem (1936) have used immiscible phenol and water mixtures in the concentration of the pernicious anaemia factor and Greene & Black (1937) and Warburg & Christian (1938) have used substituted phenols and water or salt solutions in the purification of riboflavine and of the prosthetic group of the amino-acid oxidase; these substances cannot, however, properly be included in our category of "large molecules".

A useful list of solubilities of large molecules in various organic solvents has been published by Morgan (1937), but comparatively little use has been made of such systems in the fractionation, as opposed to the preliminary extraction, of proteins and polysaccharides. The separation of liquid layers has often been noticed during fractionations, e.g. with the polysacchride of type II pneumococcus (Heidelberger & Avery, 1924), with protamines (Kossel, 1898) and with the product of partial hydrolysis of *Brucella melitensis* (Miles & Pirie, 1939*d*), but the property has seldom been used either for rigorous purification or for characterization.

Bawden & Pirie (1937*a*) observed the separation of immiscible layers, one of which was liquid crystalline, from preparations of three strains of tobacco mosaic virus that had been subjected to purification procedures that cause aggregation, and they used the limiting dilution at which the liquid crystalline layer would separate at room temperature as an index of purity. Similar layers separated in preparations of two cucumber viruses and two strains of potato virus "*X*" (Bawden & Pirie, 1937*b*,

1938 *a*). When the infectivities and immunological activities of the materials in these two layers were compared it was found that they were identical if the preparation of tobacco mosaic virus had been purified by ultracentrifugation, whereas with cruder preparations the lower layer was more active serologically, though not necessarily more infective, than the upper one. The concentration of the liquid crystalline layer when at equilibrium with the upper layer and the difference in concentration between it and the upper layer was found to depend on the purity of the preparation; each was increased by the presence of impurities, so that, as fractionation proceeded, the difference in concentration and so in specific gravity became so small that separation became slow and the recognition of the existence of two phases difficult. It appears therefore that there is a limit at which the two phases cannot co-exist except in the presence of a third component that is unevenly partitioned between the phases and that the separation of two phases, useful initially as an index of purity, might be used later as evidence for the presence of a contaminant. These relationships suggest the possibility of analysis in terms of the Phase Rule, but until the theory of the nature of the forces that are concerned in tactoid formation, for the liquid crystalline layer is itself formed by the coalescence of tactoids (Langmuir, 1938), is more clearly understood this would be unprofitable.

In other studies on the equilibria between phases it is well known that different impurities have markedly different effects on the partitioning. These differences are well illustrated by the layers formed by plant-virus preparations. Small quantities of salts do not effect the separation of layers in this case, although Langmuir (1938) has found that they inhabit tactoid formation in the analogous cases of vanadium pentoxide and benzopurpurin. The contaminants that most effectively impeded layer formation were found to be the breakdown products of the virus whether made by heating or by drying. By comparison other strains of virus and virus which had been rendered non-infective although still serologically active (by treatment with ultra-violet light for example) were relatively inert. It is clear, therefore, that differences that are serologically or phytopathologically significant may have comparatively little effect on the separation of phases. This conclusion is not necessarily incompatible with the Phase Rule, for, although from this point of view the addition of another component to a two-phase system must add one to the number of degrees of freedom, it is reasonable to assume that the addition of particles of identical cross-section (Bawden *et al.* 1936) would have only a very small effect on a tactoid. Freundlich *et al.* (1933) have found, however, that independent tactoids, i.e. a three-phase system, are formed when the tactoid-forming materials are very dissimilar.

## VIII. EVIDENCE FROM SPECIFIC SEROLOGICAL REACTIONS

It has already been pointed out that serological evidence enables us to discriminate between substances that seem to be identical by the other tests applied. This method of discrimination can only be used if the substances are prepared from independent sources so that cross-precipitations can be carried out. Anaphylactic and precipitin reactions can also give valuable information when the nature of a

possible impurity is suspected, even when only one type of preparation of the substance that is being isolated is available.

A bacterial antigen may contain materials derived from the culture medium on which the bacterium was grown, and it may be contaminated with materials which have been added for various reasons during the purification. When these substances are of small molecular weight little difficulty is experienced in their removal, for it is effected by the ordinary methods of purification such as dialysis. With reagents of larger molecular weight this is less simple. Delafield & Smith's (1939) demonstration that some properties which were supposed to be those of the specific substance of *Salmonella aertrycke* were, in fact, due to constituents of the trypsin preparation used in the fractionation is a case in point. Similarly, the agar which is apt to contaminate bacterial polysaccharides when the bacteria have been grown on agar has caused considerable confusion (cf. Sordelli & Meyer, 1931 *a*, *b*; Zozaya & Medina, 1933); Morgan (1936) has stressed the value of anti-agar antisera in testing products of this type. With virus preparations the danger is from other viruses and from normal plant or animal proteins; the former are automatically tested for if the infectivity of the final product is measured on the same species as that from which the preparation was made. Chester (1936, 1937) has used the anaphylactic response for demonstrating the presence of normal proteins in crude plant-virus preparations. In the same way the inhibition of an enzyme action by an antiserum, made by the injection of a preparation of the same enzyme derived from a different animal or plant species, would be good evidence for the identity of the two substances.

## IX. EVIDENCE FROM MEASUREMENTS OF ACTIVITY AND OF PRECIPITATION OR INFECTION END-POINTS

The types of evidence so far discussed have been criticized in terms of the degree of difference between the components of a mixture that is significant or that is large enough to be detectable by the measurements that are being made. The validity of the evidence now to be considered depends on theoretical considerations.

There are several difficulties in the use of measurements of enzymic activity, pharmacological activity, serum precipitation end-point or infectivity as an index of the purity of a preparation. In the first place it is common experience that many preparations become less stable when they have been highly purified; in such cases substances, generally looked upon as contaminants, are necessary for full activity. If these normal stabilizing materials could be replaced by other substances, preferably of known composition as in the stabilizing of enzymes or antibodies by glycerol or sucrose, it could be concluded that they were inessential and were therefore contaminants. In the same way if the prosthetic group of an enzyme is readily dissociable the distinction between a prosthetic group and a co-enzyme becomes uncertain (cf. Warburg, 1938; Dixon & Zerfas, 1940). Such an enzyme can only show full activity if the concentration of some other substances in the environment is maintained at a suitably high level.

The changes which a substance suffers in the course of manipulation generally lead to a decrease in its activity, but, as has already been pointed out, activity may

be increased. Agren & Hammarsten (1937 *a*), for example, claim that they can split off ten out of the forty or fifty amino-acids in the apparently homogeneous protein secretin without any loss of activity. This process, which results in the same activity being manifested by a smaller weight of material, should, by this criterion, be looked on as a purification, although the final product is chemically different from the starting material.

In theory it should be possible to argue about the purity of an enzyme preparation from data on the reaction velocity, the collision frequency between enzyme and substrate molecules in solution and the length of the chain, if a chain reaction is being catalysed. It is apparent, however, from the papers of Haldane (1931) and Moelwyn-Hughes (1937), to take only two examples, that the underlying theory is still too nebulous to be of value in this connexion, and that measurements of reaction velocity can only be used for ascertaining whether products from different sources may be identical.

Within a group of substances with similar properties and particle weights, e.g. the specific soluble substances of bacteria or the plant or animal viruses, valuable evidence on the course of the purification can be got from comparisons of the serum precipitation end-points at different stages. The final value attained is also of significance, for a survey of the published results shows that most highly purified products have given end-points of 1,000,000 to 10,000,000. A simple limit is set to the serum precipitation end-point of a pure antigen by the fact that a precipitate is likely to be invisible under the ordinary conditions of testing if it weighs less than $\frac{1}{1000}$ or $\frac{1}{100}$ mg., depending on its refractive index and texture. The weight of antigen that is needed to give this weight of precipitate will depend on the insolubility of the antigen: antibody complex, that is, on its tendency to aggregate into a few large flakes rather than into a large number of particles that are individually too small to see. It will also depend on the amount of antibody that is precipitated by a given amount of antigen and on the co-precipitation of other components of the system. The last is an important factor where unstable systems such as plant sap are concerned but is naturally less important with purified preparations. Hooker & Boyd (1937) and Boyd and Hooker (1934) have studied the ratio of antigen to antibody in the precipitate for various ratios of antigen radius to antibody radius, and they find that the results agree to within the rather wide limits of experimental error with the hypothesis that the antigen is covered with a layer of antibody one molecule thick. The equation has been simplified by How (1939) who, using ball bearings, has also compared the calculated figures with those found experimentally. The relevance of these observations will become uncertain if Kabat's (1939) observations on the elongation of antibody particles are confirmed; arguments from first principles will become even more difficult in cases where both antigen and antibody are highly anisodimensional. It is however clear that smaller particles precipitate a larger proportion of antibody and should therefore give higher end-points than large particles. The antigen from *Brucella melitensis* can exist in several states of aggregation with weights varying from 0·1 Mh. to about 50 Mh. and it has been found by Miles & Pirie (1939 *a*, *b*) to illustrate this phenomenon. Disaggre-

gation, which is accompanied by the loss of 5–10 % of material only, causes a tenfold increase in the precipitation end-point.

The influence of the texture of the precipitate on the visibility of a small amount of precipitate has been discussed by Bawden & Pirie (1938 *b*). Preparations of tomato bushy stunt virus appear from the work of McFarlane & Kekwick (1938) to be as homogeneous as any other protein preparations that have been made and they are certainly more homogeneous than the other plant virus preparations that have been studied. Nevertheless, the serum precipitation end-point was never greater than $1:10^6$, whereas tobacco mosaic virus preparations precipitate at $1:10^7$, potato virus "*X*" preparations at $1{\cdot}7:10^7$ and even potato virus "*Y*" preparations for which no claims of purity have been advanced (Bawden & Pirie, 1939), precipitate at $1:10^6$. The bushy stunt precipitate, unlike those given by the other virus preparations mentioned, resembles the somatic or "O" type of agglutination observed with *Salmonella* bacteria, that is, it packs into compact heavy particles that are more difficult to see than the loose, flocculent particles formed during the "H" agglutination of flagellated bacteria. This effect of the character of a serum precipitate on its visibility is presumably responsible for the fact that many plant virus preparations precipitate at considerably higher end-points than those predicted, from apparently valid premises, by Merrill (1936).

With infective preparations the end-points give us the weight of material that is needed to cause a lesion or infection. There are three factors involved in this weight; the weight of the constituent particles, the purity (that is the proportion of these particles that is infective) and the number of infective particles needed to cause whatever response is being observed. If the second two factors are disregarded these measurements give a maximum value for the particle weight; Schramm (1939) has found by this method that *Myxomatosis* virus weighs 420 Mh.

When the infective particles are visible, i.e. with bacteria, the number needed to cause infection is easily ascertainable and it is seldom found that one will suffice. The position is complicated by the enhancing effect which certain substances, notably mucin, have on the virulence of several organisms. The effects found by Nungester *et al.* (1932) with pneumococci and streptococci were relatively slight, but Miller (1935) and Anderson & Oag (1939) have found larger effects with the meningococcus, while Rake (1935) and Henderson & Morgan (1938) could reduce by a factor of $10^5$ or $10^6$ the number of typhoid bacilli in the infective dose by suspending the organisms in mucin. The increase in the infectivity of plant virus preparations that can be brought about by adding substances such as carborundum powder (Bawden, 1936) to the infective fluid is not a comparable phenomenon. In the one case the mucin probably protects the bacterium against the lethal mechanisms of the host tissue; in the other the powder apparently increases the number of points of entry of the virus into the plant tissue. With many plant virus preparations, e.g. potato "*X*" (Loring, 1938; Bawden & Pirie, 1938 *a*) and potato "*Y*" (Bawden & Pirie, 1939) the process of purification is accompanied by a loss of infectivity which is not paralleled by the loss in serum precipitation end-point. It is possible that part of this loss is due to the separation from the virus of substances acting in a manner

analogous to mucin in the cases quoted. The importance of these observations lies in the possibility that such an activating substance may be inadvertently added to a preparation during the purification, by treatment with an enzyme preparation for example, and that its enhancing effect may then be mistaken for a removal of inert material.

The possibility of getting a 1:1 relationship between infective particles and lesions will depend on the methods of infection used. Where, as with plant viruses, these are crude, an enormous wastage of material is to be expected and this is confirmed by the apparent need for $10^5$–$10^6$ particles to cause one lesion (cf. Sheffield, 1939). In the statistical treatment of the effect of virus dilution on number of lesions that Youden *et al.* (1935) have published a factor representing the maximum number of lesions obtainable on a leaf is introduced to cover this wastage, and it is this mainly that invalidates Robbins's (1934) somewhat depressing conclusion that 600,000 tons of tobacco sap would be needed to get 0·1 g. of tobacco mosaic virus. In studies on the bacteriophages and on animal viruses this difficulty is to a large extent overcome, although, as Sprunt (1939) has found with vaccinia, there may be wastage of virus if the volumes used for inoculation are too large. Several statistical treatments of the relationship between the number of particles required to cause an infection and the variations in the percentage of infections caused by doses of different size have been made along the lines elaborated by Holvarson & Ziegler (1933). Feemster & Wells (1933) and Parker (1938), using a bacteriophage and three strains of vaccinia virus, have shown that deductions from the hypothesis of effective infection by one particle give the best fit with the observed results. From the chemical standpoint it is more important to know the proportion of particles that is infective rather than the number of infective particles needed to cause a lesion, and these conclusions naturally shed no light on this question. Smadel *et al.* (1939), using values for the weight of a vaccinia elementary body derived from measurements of the sedimentation constant made by Pickels & Smadel (1938), concluded that between 2·4 and 9·2 particles caused a lesion on the skin of a rabbit. In both these papers the possibility of aggregation is recognized. McFarlane *et al.* (1939) and Hopwood *et al.* (1939) have shown that ultrasonic or mechanical emulsification of dried or flocculated vaccinia virus causes an increase in its infectivity and they showed by microscopical examination that preparations treated in this way resembled fresh preparations closely. Similar effects of aggregation have been observed with a staphylococcus bacteriophage, for Northrop (1938) finds that the particle can vary in weight from 0·5 to 300 Mh. Kalmanson & Bronfenbrenner (1939) produce evidence that a coli bacteriophage has an even smaller minimum particle weight, but that in the natural state it is absorbed on to bacterial detritus of variable size.

If an infective agent can exist in several states of aggregation it is obvious that the activity will depend on the state aggregation. Such an effect has been postulated by Bawden & Pirie (1937*a*) for tobacco mosaic virus, and it offers a possible explanation of the activating effect of X-rays that Kausche & Stubbe (1938) found with this virus. Similarly, the absence of an increase in infectivity is, as Bawden

(1939) and Stanley & Lauffer (1939) have pointed out, the best evidence that tobacco mosaic virus is not turned into a form with infective particles weighing only 0·1 Mh. by exposure to concentrated urea solutions as Frampton & Saum (1939) have claimed. In the same way any substance in a preparation which increases or diminishes the tendency of a virus to aggregate will affect its infectivity, and the removal or addition of such a substance might be interpreted as a destruction or purification of the virus.

## X. CONCLUSIONS

The conclusion drawn from this survey is that "purity" is a concept that has no meaning except with reference to the methods and assumptions used in studying the substances that are being discussed. The question "Is this pure?" is as meaningless over a large region of experience as the question "Is this alive?" is over an adjacent region. This point of view has been presented, partly by design and partly of necessity, in an unphilosophical manner in the hope that other "sooty empirics" (Boyle, 1680, p. 24) may be saved time searching the literature when it becomes necessary to make some positive statements about a product. It is probable that the question could also be approached profitably from a philosophical standpoint, and it is obvious that the apparent antithesis between a pure substance and a mixture of similar substances fits easily into the "dialectical materialist" system (cf. Haldane, 1938), but it is unlikely that an approach along these lines would be of immediate use to the workers in this field.

In a previous essay (Pirie, 1937) under the title "The meaninglessness of the terms 'life' and 'living'", some similar comments were made on nomenclature in a case where words in apparent antithesis merge their meanings smoothly with one another through a large zone of uncertainty, and it was contended that in this case the concepts themselves were at fault. The approach in the present review has been from the standpoint of the deficiencies of the criteria rather than the inadequacy or fluidity of the concept, for the concept is likely to continue to be used even although invalid. This usage will be altered, and it may become more reasonable to criticize the concept, as more investigations are carried out on the substances lying in the region of uncertainty. A few antitheses have been evaded or resolved by an arbitrary definition. This solution, however, is possible only in cases where the antagonism involves one or a few parameters as in the distinction between molecules and particles.

An acceptance of the fundamental position that purity is a property of a substance which cannot be demonstrated makes it necessary to consider carefully the phraseology used in describing preparations. A mean must be struck between pedantry and over-statement, and it would perhaps be pedantic to insert qualifications when speaking of such products as insulin. With other products, such as plant viruses, caution is fully justified. In this review and in previous publications the phrase "virus preparations" has been used, and this seems to express the element of doubt that is present better than the often-used phrase "virus proteins", which is either redundant or over-precise. A properly guarded statement cannot afterwards be

simply described as right or wrong. At worst the writer can only be accused of having conveyed a false impression and not of having made a misstatement. At best he can afterwards claim credit for his safeguarded statement only in so far that any hypothesis compatible with the facts is creditable if it is both plausible and useful. It is therefore important to avoid praising "courage" as if it were a scientific virtue when too often what is called courage would be better described as "over-confidence in the making of unproved statements"; the fact that these statements sometimes, by accident, turn out to be correct is irrelevant. This excursion into scientific ethics as opposed to methodology and terminology is perhaps justifiable when we consider how much acrimony develops over polemics and how much space is taken up by claims of priority in scientific journals.

## XI. SUMMARY

The limits, in weight and chemical stability, of the group of substances referred to as large molecules are discussed and the range is arbitrarily taken as from particles unresolvable by the microscope to those with molecular weight 5000. A unit of molecular weight is proposed and its advantages are discussed in cases where the nature of the bonds holding a particle together is uncertain.

Four distinct meanings that can be carried by the word pure are described, and examples are given of each type of purity.

The types of observation generally made and presented as evidence that a substance is pure are considered under seven headings. Chemical analysis and crystallinity are the least reliable, and specific serological tests can only show the absence of contaminants whose presence is suspected. The measurement of end-points, whether by serum precipitation or infection, is subject to several errors and can at best only indicate whether or not the major constituent of a preparation may be serologically active or infective.

Electrophoresis, ultracentrifugation and studies based on the principles of the Phase Rule, i.e. solubility and partition measurements, give the most reliable evidence of purity at present available. Several reasons are given for thinking that even these methods may be fallible.

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# THE PROCESS OF REGENERATION IN HYDROIDS

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(*Received* 27 *February* 1940)

CONTENTS



## I. INTRODUCTION

Regeneration of a structure is essentially an embryonic process which involves the reorganization of a group of cells into a new form. However, whereas in typical embryonic development the sperm acts as the initial stimulus, in regeneration, cutting or otherwise exposing a group of cells to the action of the environment serves as the initial stimulus. After the stimulus has been applied, the response of the tissue varies according to a number of well-established factors. It is these two major problems of regeneration, (1) the nature of the stimulus, and (2) the factors governing the response of the tissues, which form the subjects of this review. For the sake of simplicity and uniformity only regeneration in the hydroids or closely related forms will be considered.

## II. THE NATURE OF THE STIMULUS FOR REGENERATION

The cutting of a hydroid stem has long served as a stimulus for the regeneration of a hydranth, but the exact nature of this stimulus was not known. Cutting off a part does two things: first, it releases the cells at the cut surface from the physical and chemical influences of the part removed, and secondly, it creates a new external environment for these cells. This new environment may involve a number of factors. (1) Some cells at the cut surface may be injured, giving rise to "wound hormones" which might stimulate growth and differentiation. (2) The

exposed cells are able to lose substances more readily, possibly inhibitory substances which prevent regeneration. (3) The exposed cells are in direct contact with the oxygen in the medium. (4) They are exposed to the direct action of salts in solution.

The removal of the hydranth in *Tubularia* is not in itself sufficient to cause regeneration, since if a ligature is tied through the stem after cutting off the hydranth, regeneration is inhibited. Evidently the perisarc blocks some factor that is necessary for regeneration, and the tissue must be exposed directly to sea water.

Sea water is not the necessary factor, however, since Morgan (1903) showed that cut ends of *Tubularia* placed in sand in sea water could not regenerate. Barth (1938*a*) found that a glass capillary placed over the cut end would inhibit regeneration. These experiments indicate that interference with the free circulation of sea water around the cut surface stops the process of regeneration. This interference might be with the conveyance of materials *from* the cut surface or with the conveyance of oxygen *to* the surface.

That oxygen is an important factor is shown by experiments of Torrey (1912), Miller (1937) and Barth (1937, 1938*a*). Miller found that when one end of the stem was bathed with oxygen in sea water and the other end with boiled sea water, the hydranth formed at the end in contact with oxygen. Further experiments showed that circulating sea water stimulated more hydranths than did standing sea water. Finally, the stimulating effect of circulating sea water was not simply due to agitation and removal of carbon dioxide, because experiments in which nitrogen was bubbled through sea water showed complete inhibition. Circulating sea water is not necessary for regeneration, however, as regeneration may be very rapid in standing sea water when the oxygen tension is high (Table I).

Table I. *Effect of varying oxygen tensions on the rate of regeneration of* Tubularia

Exp. 7: 180 young stems, 5–7 mm. in length.

| Oxygen c.c./litre | Length of hydranth primordium ($\mu$) | Time for primordium formation (hr.) | Rate of regeneration (length/time) |
|---|---|---|---|
| 2·4 | 1072 | 36·1 | 29·7 |
| 3·2 | 1284 | 28·1 | 45·7 |
| 4·1 | 1370 | 26·8 | 51·3 |
| 4·8 | 1365 | 26·3 | 52·0 |
| 8·2 | 1640 | 24·5 | 67·0 |
| 11·3 | 1809 | 24·6 | 71·8 |
| 14·3 | 1840 | 24·1 | 75·5 |
| 16·5 | 1846 | 23·7 | 77·0 |
| Control in open dish | 1370 | 26·2 | 52·2 |

The experiments of Barth (1937, 1938*a*) explain the above results on the basis of varying oxygen tension at the cut surface. First of all the rate of regeneration is directly dependent upon the oxygen tension (Table I). Secondly, when the oxygen tension is less than about 1 c.c. per litre the process of regeneration is stopped, although the stems remain alive and will regenerate if returned to normal sea water. Since hydranths do not develop at a cut surface when the perisarc is tied over the

surface it is reasonable to suppose that the perisarc interferes with the circulation of oxygen to the cut surface just as the glass tube does. If this is true, then a removal of the perisarc might allow regeneration by bringing the tissues in direct contact with the oxygen of sea water.

Child (1927*a*, 1929*a*) showed that hydranths could develop from the side of the stem of *Corymorpha* through injury of the stem and removal of the dominant apical hydranth. Zwilling (1939) was able to demonstrate that in *Tubularia* exposure of the stem without cutting was sufficient to start regeneration. By simply cutting out a piece of the perisarc from the middle of the stem the exposed tissue will form two hydranths, one on each side of the opening. Since no cut is made through the stem the two hydranths remain connected by their oral ends. Here it is clear that the stimulus is not one of cutting or injuring the tissue, but simply exposing the uncut stem to sea water. This can only mean that either some inhibitory substance escapes from the free surface or that an increased supply of oxygen stimulates. That an inhibitory substance escapes is unlikely from Miller's (1937) experiments, where at one end oxygen was bubbled through the sea water while at the other end nitrogen was used. At both ends a vigorous circulation was set up, removing any inhibitory substances, yet only at the oxygen-treated end did regeneration take place.

Yet it might be argued that both the removal of inhibitory substances and the presence of a sufficiently high oxygen tension were necessary for regeneration. Recent experiments show definitely that it is oxygen alone that is responsible for the initial stimulus for regeneration in *Tubularia*. Tying off both ends of the stem, Rose (1940) injected oxygen gas into the coelenteron and the stem began to regenerate. In some stems regeneration occurred at the distal end, even though the perisarc was tied over the end. This end would be expected to regenerate since, if all of the stem is exposed to high oxygen tensions, the distal end having the greatest capacity for regeneration will become dominant. Other cases are even more significant. When the injected oxygen breaks up into a number of small bubbles a region of regeneration is set up about each bubble, and if the stem is cut at these regions a hydranth forms in a short time.

Since in the above case there was no exit for the escape of inhibitory substances, the sole factor stimulating regeneration was the introduction of oxygen into the system. We have then the interesting situation that any part of the stem will form a hydranth if the oxygen supply is increased sufficiently. There is no need to postulate wound hormones or inhibitory substances. Cells will organize into a hydranth in relation to an incoming supply of oxygen with the oral end of the hydranth at the highest oxygen tension, as seen by the position of the oral ends of two hydranths in Zwilling's experiment quoted above.

A further extension of the role of oxygen is found in the experiments of Child (1928) dealing with the reorganization of aggregates of dissociated cells of *Corymorpha*. After grinding the stems in sand and filtering, the cells were collected in an amorphous aggregate. These, if left undisturbed, form hydranths from the upper exposed surface while stolons form at the attached surface.

Likewise, Child (1927*b*) found that short stems in which polarity was obliterated by means of alcohol and other inhibiting agents formed hydranths on the upper surface. When returned to normal sea water as many as five hydranths might form from one stem. Child suggested a "difference in respiratory exchange" as the factor determining the new polarity. It seems clear that the higher oxygen tension at the exposed surface is responsible for the development of the hydranth just as in the case of the aggregates of dissociated cells.

The manner in which oxygen acts is suggested by experiments in which the oxygen tension of sea water is varied and the oxygen consumption measured along with rate of regeneration (Barth, 1938*a*, 1940). It was found that as the oxygen tension was raised above that of sea water an increase in oxygen consumption of the stem resulted with an accompanying increase in rate of regeneration. When the oxygen tension was decreased the oxygen consumption fell off and rate of regeneration fell to zero.

The suggested mechanism for the formation of a hydranth from a given region of the stem is outlined as follows. The tissue of the stem will not form a hydranth within the perisarc because the oxygen supply is low, the oxygen consumption being too low for the process of regeneration. Any treatment that will increase the rate of oxygen consumption, such as removing the perisarc, or injecting oxygen gas, or even shaking vigorously (Barth, 1940), will start regeneration. The parts of the hydranth form in relation to the increased oxygen supply with the oral end at the highest oxygen tension.

The experiments of Goetsch (1929) can be explained by this mechanism. This investigator showed that the stem of *Cordylophora* would grow inside a glass tube until it came to the opening where it would differentiate into a hydranth. Likewise, in *Pennaria*, when the cut ends of the stem were all sealed, a hydranth would differentiate at an artificial opening made in the perisarc.

The experiments of Child (1927*a*, 1929*a*) in *Corymorpha* require some additional explanation. In this form, which has most of its stem free of perisarc, it might be asked why regeneration does not occur all over the surface. Here the factor of dominance of the distal hydranth or distal regenerating end prevents the formation of a hydranth unless an extensive injury is made. We must assume then that the injury in some way sets up a region of high enough oxygen consumption to compete with the distal end of the stem. This could occur by the exposure of a greater surface to oxygen by the cut or by the stimulating effect of injured cell products on the respiratory mechanism. In view of the fact that cutting is not necessary in *Tubularia*, but only the increased availability of oxygen, the former explanation is the more likely one.

## III. THE RESPONSE OF THE TISSUE TO THE STIMULUS

First of all, it is well established that any group of cells, if large enough, organizes itself into a hydranth. The experiments of Child (1928) on *Corymorpha* show that a mixture of cells produced by grinding the stems with sand will form a hydranth and stolon if left undisturbed. Similar experiments have been reported

by Issajew (1926) and Weimer (1934) on *Hydra*, and Beadle & Booth (1938) on *Cordylophora* and *Obelia*. Further, the early workers showed that sections of the stem at any level would regenerate. Still more significant is the fact that a hydranth can form at both the distal (apical) and the proximal (basal) ends of a segment. Thus there can be no question of predetermined regions destined to form any particular structure or of a fixed polarity of the stem. The determination of a hydranth takes place by the interaction of several factors.

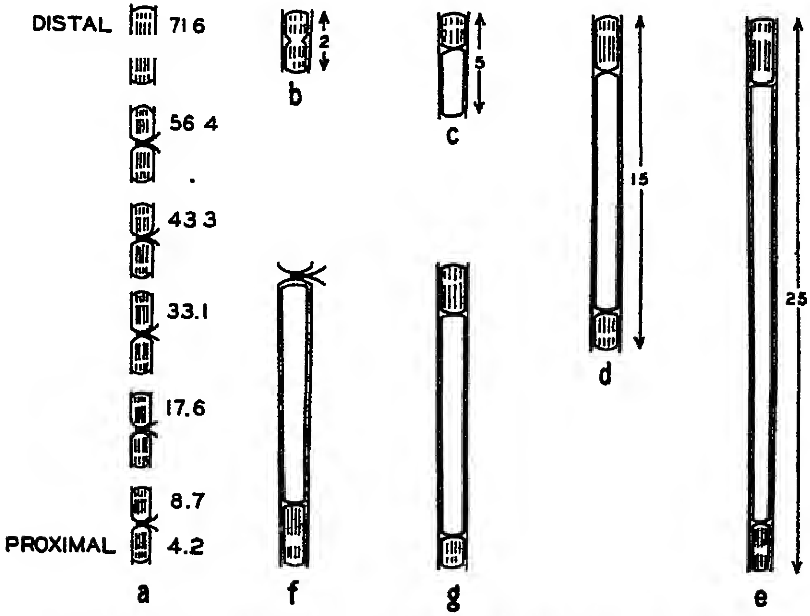


Fig. 1. Graded rates of regeneration and dominance of the distal regenerant of *Tubularia*.

*a*. Rate of regeneration falls off from distal to more proximal levels of stem (Barth, 1938*b*). Numbers give rates of regeneration in $\mu^3$/hr. × $10^5$. These rates are inherent in the stem and have not been influenced by dominance, which is blocked by a ligature; variations in rate are assumed to be caused by variations in concentration of some catalyst.

*b, c, d, e*. Typical response of varying length of stem. Shortest stems (*b*) are bipolar, then complete dominance (*c*) of distal end, and partial dominance (*d, e*) as stem increases in length. (Cf. Table II.)

*f, g*. Method of measuring dominance of distal regenerant. In *f* distal end is ligatured and regenerant inhibited, so that proximal end regenerates independently (absolute rates (*a*), Table II). In *g* distal end regenerates, and proximal end is retarded in development or completely prevented from regenerating according to length of stem (relative rates (*r*), Table II). Dominance is measured as percentage retardation in rate of regeneration, and varies from about 7 to 100 % (Barth, 1938*b*).

## (1) *Regional differences along the stem*

The first of these factors is the physiological gradient in the stem as exhibited by the graded rates of regeneration at various levels of the stem. These regional differences were pointed out by Driesch (1899), Child (1907) and others. Recently, Barth (1938*b*) has shown how the rate of regeneration varies with level of stem (Fig. 1).

### (2) *Dominance in regeneration*

The second factor affecting regeneration of the hydranth is physiological dominance (Child, 1929*b*). In the first place the distal hydranth exerts dominance over the stem so that a proximal cut end will not form a new hydranth unless the distal hydranth is removed (Watanabe (1935) in *Corymorpha*). Likewise, Child (1932) found a reduction in the number of hydranths stimulated by cutting and by transplantation when the distal hydranth was present. Dominance also controls lateral budding (Child, 1929*b*). A second form of dominance is that exerted by a distal cut end over a proximal end of any isolated segment of the stem. (See Child (1929*b*) for general discussion and earlier investigations.) Fig. 1 *b*, *c*, *d*, *e* shows the situation in *Tubularia*. This dominance has recently been measured (Barth, 1938*b*) by comparing the rates of regeneration of the proximal end with and without the presence of a regenerating distal end. The distal end is prevented from regenerating by means of a ligature (Fig. 1*f*). Table II shows the percentage dominance exerted by the distal regenerating end over the proximal end of various stems. In general, the shorter the stem the greater is the inhibiting effect. However, even in very long stems there is some inhibition.

Table II. *Comparison of relative and absolute rates of regeneration of* Tubularia.

$D^r$, relative, $D^a$, absolute rate of regeneration of distal end; $P^r$, $P^a$, relative and absolute rates of proximal end in $\mu^3$/hr. $\times 10^5$. Dominance $= \frac{P^a - P^r}{P^a} \times 100$ (see Fig. 1 *f*, *g*).

| Exp. | No. stems | Length mm. | $D^r$ | $P^r$ | $D^a$ | $P^a$ | Dominance % |
|---|---|---|---|---|---|---|---|
| A | 6 whole stems | 5 | 45·5 | 0 | — | 36·8 | 100 |
| B | 14 distal halves | 7·5 | 40·5 | 0 | 38·6 | 31·9 | 100 |
| C | 14 proximal halves | 7·5 | 23·4 | 3·9 | 24·9 | 15·1 | 74 |
| D | 10 distal thirds | 10 | 52·7 | 10·7 | 57·0 | 35·9 | 70 |
| E | 10 middle thirds | 10 | 36·1 | 4·4 | 26·0 | 13·0 | 66 |
| F | 10 proximal thirds | 10 | 19·1 | 0 | 27·0 | 8·6 | 100 |
| G | 10 distal halves | 13 | 86·7 | 41·7 | 90·5 | 53·0 | 22 |
| H | 10 proximal halves | 13 | 41·4 | 17·9 | 53·6 | 28·5 | 38 |
| I | 10 whole stems | 15 | 67·0 | 12·6 | 63·0 | 26·8 | 54 |
| J | 10 whole stems | 15 | 46·7 | 18·8 | 40·4 | 20·4 | 7 |
| K | 10 whole stems | 20 | 49·5 | 19·0 | 51·2 | 28·9 | 34 |
| L | 10 whole stems | 25 | 105·5 | 29·0 | 108·0 | 47·4 | 39 |
| M | 10 whole stems | 25 | 72·5 | 27·9 | 66·8 | 34·0 | 20 |

### (3) *Size and regeneration*

Selecting stems of *Tubularia* of the same size and isolating from them sections of varying length, it is found that the larger the stem the larger the regenerant (Hyman, 1926; Child, 1931; Barth, 1938*b*).

### (4) *The mechanism of dominance*

Two views concerning the general phenomenon of dominance have been suggested by various investigators. One of them is that dominance is a factor that is transmitted through cells, while the other view utilizes the transport of substances. These views are discussed in general by Child (1929*b*).

With specific reference to the hydroids, the transmissive theory has been supported by Hyman (1920), Child & Hyman (1926), Hyman & Bellamy (1922), and especially Lund (1922, 1925) in *Obelia*. The evidence is based on a correlation between the electrical differences in potential and the inhibiting effect of externally applied currents. The argument is that if an electrical difference of potential is set up between a regenerating region $D$ and some other region of the stem $P$, the current flowing may be great enough to inhibit regeneration at the region $P$.

Barth (1934*a*, *b*) found variability in both the potential differences in various hydroids and also in the inhibiting effects of an externally applied current. A correlation between the two phenomena was found, but its significance in explaining dominance was questioned. Recently, Burr & Hammett (1939) have again made a preliminary survey of the potential differences in *Obelia*.

The fact that organisms show patterns of electrical differences in potential is not in itself any evidence that these potentials have anything to do with the development of this pattern. Indeed, it is unthinkable that the stem of a hydroid, with its cells containing electrolytes and having semi-permeable membranes, would *not* show electrical differences in potential. And further, since the cells do not form a homogeneous system, it follows that there must be some sort of pattern formed by these potential differences. Then, too, it is impossible to conceive of any living system which would not be affected by passing an electric current through it. Therefore these two phenomena are merely necessary results of the organization of any living system, and their existence is not in any way significant to the problem of dominance.

These two phenomena must be causally related in order to use them to explain the patterns formed by growth and differentiation. Thus far it has not been demonstrated that the growing or differentiating hydranth sets up a potential difference which would cause a flow of current of sufficient magnitude and for such a length of time as to inhibit regeneration at other levels of the stem.

The view that dominance is exerted through transport of substances has received some support recently by Barth (1938*b*). It was found that certain phenomena could be explained on the basis that the two cut ends of a stem competed for materials circulating in the stem. The two ends of stems were isolated by ligatures as in Fig. 2*a*, *c*, *e*, and the rates of regeneration (initial rates) were compared with the relative rates in stems in which the two ends were competing with each other, as in Fig. 2*b*, *d*, *f*. With ligatures, the ends of the stem are cut off from the circulation in the rest of the stem, while without the ligatures the ends are able to use any substances in circulation. As Fig. 2*a*, *b* shows, in long stems the relative rates of regeneration (without ligature), increase proportionally to the initial rates (with ligature), indicating a simple partition of substances supplied by the middle of the stem.

As the stem is made shorter (Fig. 2*c*, *d*), so that there is less substance available, the relative rate of regeneration of the distal end begins to increase at the expense of the proximal end, so that in very short pieces without a ligature no regeneration takes place at the proximal end (Fig. 2*e*, *f*).

If substances are circulating in the stem and are used by the process of regenera-

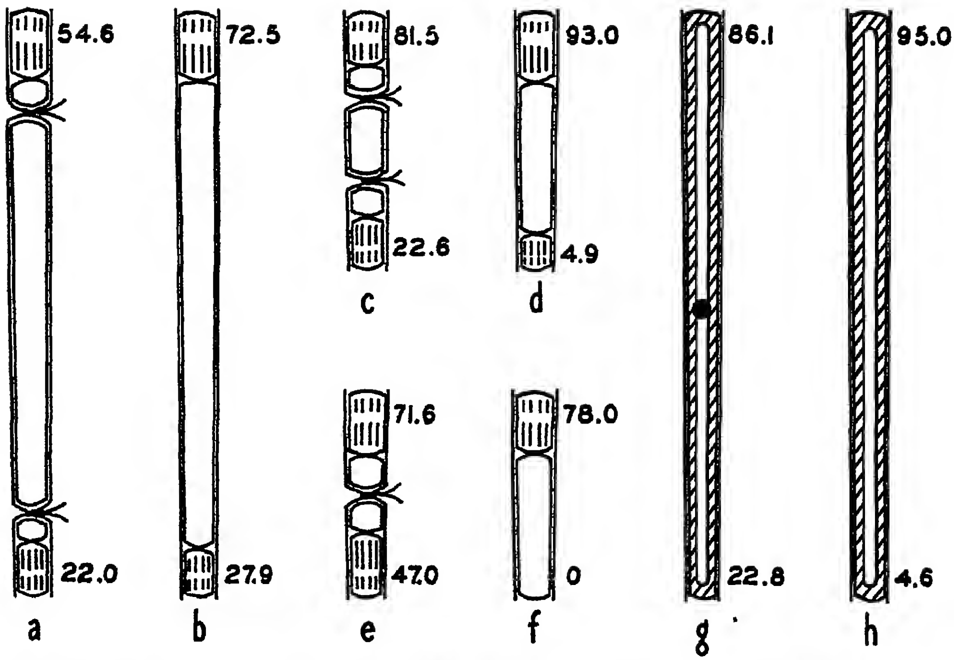


Fig. 2. Competition of the proximal and distal regenerants in *Tubularia*. The numbers give rates of regeneration in $\mu^3$/hr. $\times 10^5$.

*a, b*. Long stems. *a*. Proximal and distal ends isolated from middle of stem, compared with *b*, where proximal and distal ends compete. Both ends increase in rate in *b*, indicating a partition of substances of the stem between the ends.

*c, d*. Medium length, as above; distal end increases while proximal decreases.

*e, f*. Short stems, as above; distal end increases; proximal completely inhibited.

*g, h*. Dominance blocked by oil (in *g*) injected into coelenteron thus stopping circulation. Rate of proximal end of stem increases from 4·6 (*h*) to 22·8 (*g*), showing that distal end no longer influences rate of regeneration when circulation between these two regions is stopped.

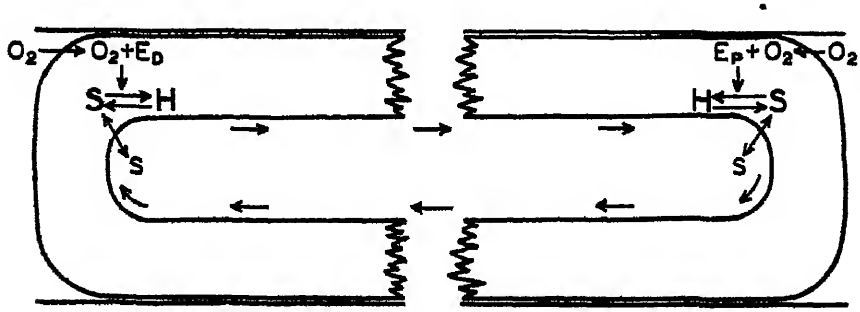


Fig. 3. Schema of competition for and partition of hypothetical substances (*S*) by distal and proximal ends of the stem of *Tubularia*. $E_D$ and $E_P$, concentrations of some synthetic factor (presumably enzymes) which controls the rate of regeneration at the distal and proximal ends; $E_D > E_P$. This factor varies in concentration along the stem according to the rates of regeneration (Fig. 1 *a*). *S*, substances necessary for hydranth formation which are present in the coenosarc and also in the circulating medium of the coelenteron. *H*, converted substances which bring about hydranth formation. $O_2$, oxygen.

tion at the ends, then stopping the circulation ought to isolate the two ends so that they would regenerate independently of one another. Blocking of the circulation by means of injection of a droplet of oil into the coelenteron showed that dominance was blocked and the proximal end regenerates independently (Fig. 2*g*,*h*).

The theory of dominance as a competition of two (or more) regions for available substances for regeneration is shown diagrammatically in Fig. 3. $S$ represents the substances involved in the formation of a hydranth, $S \rightleftharpoons H$, and is present within the cells and circulates in the coelenteron. $E_D$ and $E_P$ represent concentrations of a synthetic factor in the protoplasm (presumably enzymes) which convert $S$ into $H$ during the process of regeneration. Differences in concentrations of $E$ account for the regional differences in regeneration as observed in Fig. 1*a*. Oxygen is regarded as the external stimulus necessary for differentiation and is arbitrarily entered with $E$.

When $S$ is large, as in long stems, it is converted into $H$ at both ends according to the concentration of $E$. Thus a simple partition of $S$ between the two ends takes place as the rates of regeneration in Fig. 2*a*, *b* indicate. As $S$ becomes less, as in shorter stems, relatively more is taken up by the distal end as compared with proximal end because $E_D$ is present in greater concentration than $E_P$. In this way the concentration of $S$ is lowered in the circulation and $S$ is lost from the proximal end. Thus the rate of regeneration at the proximal end is decreased as in Fig. 2*c*, *d*. Finally, when $S$ is very small (Fig. 2*e*, *f*), it is converted into $H$ so rapidly by $E_D$ that the concentration of $S$ becomes lower than the minimum for regeneration at the proximal end.

It will be seen that a fundamental assumption of this theory is that since $E_D$ is greater than $E_P$ it is able to convert $S$ into $H$ at a lower concentration of $S$.

A further application of this theory might be made to very short stems which produce bipolar forms with a whole or partial hydranth at each end (Fig. 1*b*). This situation could be explained on the basis of $E_D = E_P$, therefore no competition and no dominance occurring.

### (5) *Underlying factors in the gradient of rates of regeneration along the stem*

Following the earlier work of Child (1926), which demonstrated gradients of susceptibility, of reduction of potassium permanganate, of the indophenol reaction, and of penetration along the stems of *Corymorpha*, further investigation by Watanabe (1935) and Child & Watanabe (1935) has shown a close correlation between the rate of reduction of methylene blue and the process of regeneration (Fig. 4). Using sections of the stem free of perisarc they found that when two regions of high rate of reduction of methylene blue were present at the two ends of the stem, both ends formed a hydranth. In some cases only the distal end showed the high rate of reduction of the dye, and correspondingly only the distal end formed a hydranth.

Gradients in sulphydryl activity were revealed by Child & Hyman (1926) using the nitroprusside reaction in the stems of *Corymorpha*. Recently, Chapman (1937) and Hammett & Chapman (1938) have shown localization of sulphydryl containing compounds and also of free amino acids at the regenerating tip of the stem and the hydranth of *Obelia*.

The experiments can be criticized on the basis that the differences in activity may be caused by the process of regeneration rather than that they precede this process. However, since the differences in these various activities are found in the resting stem, they cannot be caused by the process of regeneration but are characteristic of the physiological gradient in the stem.

The nature of the gradients found along the stem suggests that they are respiratory in nature. Actual respiratory differences in the stem were measured by Hyman (1926) in *Tubularia*, Child & Hyman (1926) in *Corymorpha*, where differences in the rate of oxygen consumption were shown to exist in parts of the stem with the highest rate at the distal end. Since the measurements were begun at from 5 to 21 hr. after cutting the stem, it is possible that the differences resulted from the regeneration process and were not present in the intact stem.

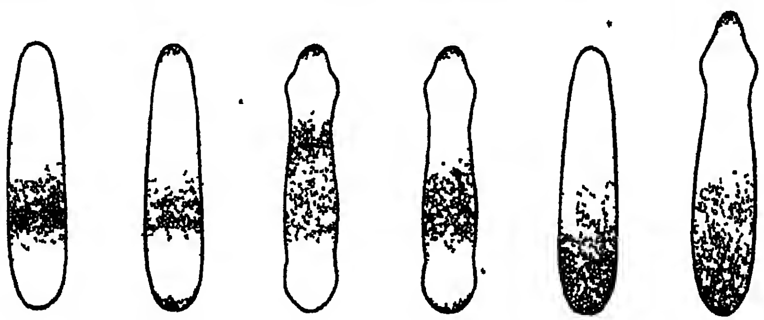


Fig. 4. Differential rate of reduction of methylene blue by the stem of *Corymorpha* (Watanabe, 1935; Child & Watanabe, 1935). *a, b, c, d.* Rate of reduction in the case of bipolar regeneration, two regions of high rate corresponding to two regions of regeneration. *e, f.* Single gradient in rate of reduction and single hydranth differentiating from most active region.

This explanation is made unlikely by the experiments of Barth (1940) in which stems were sectioned and the oxygen consumption was measured continuously with the Warburg manometer, with the result that differences in oxygen consumption along the stem appeared as soon as 1 hr. after the parts were isolated. Further, these differences in oxygen uptake between the proximal and distal segments are present in long stems in which the regenerant forms only a small part of the stem, and so could not possibly bring about the observed difference in oxygen consumption. We may take it as proven then that a gradient in the rate of oxygen consumption exists in the resting stem with the greatest rate at the distal end.

All of this work points to an underlying gradient in the concentration of substances in the protoplasm concerned with rate of respiration, or a gradient in the concentration of active protoplasm itself.

## (6) *Origin of the gradient*

Experiments of Child (1926) trace this gradient back to very early stages in development where the apical end of the planula shows a region of high activity as regards susceptibility and oxidation-reduction gradients. From other observations,

such as the position of the egg during its growth period, it is probable that the gradient arises during this early stage (Child, 1925).

A gradient may originate in masses of cells formed by aggregation of dissociated cells (Child, 1928), or new gradients can arise after the original one is obliterated (Child, 1927*b*). The masses of cells, if allowed to remain in one position, form a hydranth from the upper surface, but if moved from time to time apolar or partial forms result having no polarity. It seems clear from the evidence in § II that oxygen stimulates the exposed region of the mass to the oxidative activity necessary for the process of regeneration. Then this region exerts dominance over surrounding tissues, according to Fig. 3, by competition for some factor $S$.

Beadle & Booth (1938) are not in complete agreement with the experiments of Child. Using small pieces of the coenosarc which were united to form larger masses of tissue, they found hydranths might grow out from the side of the mass instead of the top. This result may be explained if we assume that in uniting the small pieces of the coenosarc some of the more active parts come to lie on the side of the mass and so would be most likely to form new hydranths. Both Issajew (1926) and Weimer (1934) show that in reuniting fragments of *Hydra* the distal fragments in the mass organize and form part of the new hydranth. Thus in these experiments there must be a balance between the local differences in the mass and the effect of oxygen on the mass as a whole. The experiments of Beadle & Booth, Weimer, and Issajew differ from those of Child in that the latter was dealing with very small fragments, less than $40\mu$ in diameter, which would form a homogeneous mixture in the aggregate or mass. The other investigators were using larger fragments which would form a mosaic and result in a heterogeneous aggregate or mass, where one part might be composed of very active cells and so give rise to the new hydranth. The whole question of the determination of new outgrowths could be settled by obtaining a homogeneous mass of cells and applying oxygen locally to these masses.

### (7) *Experimental modification of the stem*

Whether or not an organizer may be said to act in regeneration depends on the interpretation of the organizer in the amphibian egg. Recently, there has been a tendency to consider the amphibian organizer as a stimulus which may release something in the tissue which then brings about differentiation. Further distinctions are being made between induction of a neural tube and the organizer which induces an embryonic axis. In some of the early work these two processes were confused. In the case of the hydroid stem investigations so far have been on the induction of a whole new axis, which is comparable only to the work on the induction of secondary embryos by the living organizer.

Child (1929*a*, 1932) was able to induce a hydranth to form at any level of the stem of *Corymorpha* by lacerating the stem and also by transplants of tissue from various levels of the stem, especially distal levels. Since a distal transplant was more effective than a proximal one there was evidently some organizer action in the sense that a small piece of tissue was able to impose its activity upon surrounding tissues. Beadle & Booth (1938) found that the oral cone of *Cordylophora* induces a

hydranth when transplanted to a mass of tissue formed by the reunion of fragments of the stem.

Similarly, the results of Issajew (1926) and Weimer (1934) on the reorganization of mass of *Hydra* tissue showed that the distal fragments in the mass determined where the hydranth formed. Thus it is well established that a distal fragment of the stem is able to induce hydranth formation and thus might be termed an organizer.

The experiments of Zwilling (1939) on *Tubularia* show clearly that exposure of the tissue to sea water without cutting was sufficient for the development of a hydranth on each side of the opening made in the perisarc. Here, as previously explained, the influx of oxygen to the tissues caused them to become organized. Is oxygen therefore an organizer, or does it release an organizer in the tissue? Since oxygen not only stimulates hydranth formation but also controls the pattern of the hydranth, the oral end of each hydranth always forming at the region of highest oxygen supply, it is clear that oxygen is an organizer.

The question of what is *the* organizer in all the above cases is a futile one, since the process of organization undoubtedly entails a chain of chemical reactions, and it would be unwise to emphasize one part of the chain to the exclusion of others. Thus the organization of a hydranth begins with the physical process of cutting the perisarc, which in turn exposes the tissue to the oxygen dissolved in sea water. This oxygen next raises the oxygen consumption of the stem (Barth, 1940), and sets up a gradient in oxygen consumption for a short distance along the stem. Since oxygen diffuses in from a single opening and is used up as it diffuses, a gradient of oxygen tension must result, and so a gradient in oxygen consumption since we know that the oxygen consumption of *Tubularia* varies with oxygen tension. The hydranth then forms in relation to this gradient of oxygen consumption and the chemical products of this gradient which are as yet completely unknown.

From this discussion it is easy to see how various treatments might give the same results by acting on different parts of the chain of events. Implantation of a distal fragment which has a high oxygen consumption into the middle of the stem which has a lower oxygen consumption would result in a gradient. Likewise a cut, by exposing a greater amount of surface to oxygen, would increase the oxygen available to each cell, and the oxygen consumption would rise. Simply removing the perisarc in *Tubularia* accomplishes the same purpose. Other tissues and substances might act in the same way, but would have to be investigated individually. Not all tissues having a high rate of oxygen consumption would necessarily stimulate a hydranth as some tissues having an initially high rate of oxygen consumption might by cutting and transplantation lose this property, or they might not unite properly with the tissues of the stem to which they are transplanted.

With regard to possible action of chemical substances as organizers (Beadle & Booth, 1938), it does not follow that all respiratory stimulants would so act. The respiratory mechanism is complex and various phases can be acted upon independently. Indeed, Torrey (1933) has already shown that dinitrophenol, a known respiratory stimulant, does not have an accelerating effect on regeneration in *Tubularia*. Other substances, such as pyocyanine, methylene blue, dimethyl-

paraphenylene diamine, sodium azide, iodocetic acid, should be tried by local application, so as to set up a gradient, and also on the stem as a whole, to study influence on rate of regeneration and rate of oxygen consumption.

### (8) *External factors modifying the process of regeneration*

*Inhibition of regeneration.* Child (1931) made a study of the effect of potassium cyanide on the size of hydranth primordia in *Tubularia* and showed a decrease in size with treatment. Hyman (1920) measured the time for regeneration in cyanide and found the process to be retarded. Child (1927*b*), using a wide variety of toxic agents and anaesthetics, showed that hydranth formation could be inhibited without killing the stem. Recently, Beadle & Booth (1938) demonstrated that lack of calcium and magnesium ions suppressed the formation of a hydranth from masses of coenosarc. Similarly sodium cyanide, phenylurethane and low temperature inhibited. They found that even under these inhibiting conditions an oral cone implant would induce a hydranth.

*Inhibition and stimulation of regeneration.* Hammett, in a series of experiments with collaborators, has divided the process of regeneration in *Obelia* into six major activities. These are: Initiation, Proliferation, Differentiation, Organization, Maintenance and Catabolism, i.e. the breaking down of the hydranth and absorption by circulation (Hammett & Schlumberger, 1937). Some of these six processes have, in addition, several types. By studying the action of amino acids on these various processes Hammett was able to find specific differences between the actions of the various amino acids. Thus, whereas *l*-serine retards the process of initiation, it enhances the process of proliferation, differentiation and organization; *l*-aspartic acid is more limited to favouring the process of differentiation; while *d*-valine has no beneficial effect on the above processes but simply a retarding action on the formation of rudiments of hydranths and gonophores. Other amino acids show specific actions. (See Hammett & Toman (1939) for references to the individual papers on various amino acids.)

The effect of other agents on regeneration of hydroids has not been extensively studied, and there is great need for a comprehensive study of the effects of temperature (Moore, 1939), light (Loeb & Wasteneys, 1917), and other radiations (Strelin, 1929; Zawarzen, 1929; Puckett, 1935), and, in view of the conclusions in this review, of agents which affect the reactions involved in respiration (Child, 1931; Beadle & Booth, 1938; Torrey, 1933).

### (9) *Origin of cells in regeneration*

The old problem of the behaviour of germ layers during regeneration is as yet unsolved. All possibilities have been suggested, and the experiments give no finality. Thus the work of Kuchner (1934) Strelin (1929) and Zawarzen (1929) indicate that the interstitial cells form ectodermal and endodermal structures in *Hydra*. The work of Papenfuss (1934) and Roudabush (1933) and Beadle & Booth (1938) tend to show that the germ layers do not change during regeneration

but that extensive cell migration may occur. More work will be needed, however, before conclusions can be drawn.

## IV. SUMMARY

1. In the hydroids in general, and *Tubularia* and *Corymorpha* in particular, the stimulus for regeneration involves two factors: (1) removal of dominance exerted by either the fully formed hydranth or a regenerating hydranth, and (2) the presence of a gradient of oxygen at a localized region of the stem or a mass of cells isolated from the stem.

2. Dominance, or the inhibitory effect of a regenerating region, can be explained either on the basis of electrical differences in potential which produce chemical inhibition, or as the competition of regions of the stem for hypothetical substances circulating in the stem and necessary for regeneration. As yet no single experiment has decided between these two explanations of dominance and both should be subjects for investigation.

3. The regional differences in rate of regeneration are correlated with regional differences in the rate of oxygen consumption of the stem, and the rate of regeneration can be varied by varying the oxygen consumption of the stem. These regional differences in oxygen consumption are due to differences in the resting stem and are not caused by the regional differences in regeneration.

4. Since the stimulus for regeneration can be traced to an oxygen gradient, which results in an oxygen consumption gradient in the tissues, there is little value in speaking of an organizer in hydranth formation. Any agent which will bring about the above conditions will be an organizer. It is, therefore, possible to speak of the following as organizers: (1) oxygen locally applied, (2) tissues that have a higher oxygen consumption than the surroundings, and, perhaps, (3) respiratory catalysts, when their effects are investigated. On the whole, it would be better to speak of the coenosarc as organizing itself in response to a stimulus. The manner in which the tissue responds by cell movements and change in form is, of course, entirely unknown.

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# LACTATION

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(*Received* 5 *March* 1940)

CONTENTS



THE aim of this review is to consider from three aspects (endocrine, nervous and biochemical) the present state of knowledge concerning the physiology of lactation, a branch of reproductive physiology which has undergone notable advances in recent years. It should be understood that by the term lactation is meant the sum total of the complex of phenomena which comprise milk formation and its ejection from the gland. In this sense, then, lactation comprises milk secretion together with the events associated with suckling and milking. It may be further added that since the mammary apparatus undergoes striking changes during various stages in the life of the mammal, it is quite proper that some consideration of the development of the mammary gland should find its place in a review on lactation.

## I. THE GROWTH OF THE MAMMARY GLAND

Experimental evidence has long been in existence (for summary see Turner, 1932) which indicates that the development and secretory activity of the mammary gland is controlled chiefly by hormonal mechanisms, though, as will be seen later,

recent work indicates that nervous influences are concerned in lactation to a greater degree than was formerly supposed.

### (1) *The effect of oestrogens and progesterone*[1].

The earlier work on the experimental growth of the mammary gland will not be enlarged on here as it has been reviewed by Nelson (1936) and Turner (1939). In many species, oestrogen treatment promoted mammary duct growth only; in others, such as the rat and rabbit, a certain amount of alveolar development was also obtained. The guinea-pig was found to respond to oestrogen treatment with full mammary development. More recently the same has been shown to be true for the female goat by de Fremery (1938) and Folley *et al.* (1940), though no mammary growth has yet been obtained in males. As regards the monkey, Gardner & van Wagenen (1938) are in agreement with previous claims (see Nelson, 1936) that oestrogen treatment will cause alveolar, as well as duct development in males and females, and indeed their results indicated that with long-continued treatment (30 weeks) complete development of the alveolar system would occur. Contrary results have however been obtained by Folley *et al.* (1939) who found no alveolar development in the mammae of male monkeys which had received oestrone for periods up to one year. The female mammary gland however was more responsive to oestrogen treatment and lobules of alveoli formed in some female glands.

There is some evidence that the chemical nature of the oestrogenic substance used influences the type of mammary proliferation produced. Thus while Turner *et al.* (1932) found no differences between oestrone and oestriol as regards their action on the mammary gland, oestrone benzoate has been observed to cause alveolar development in the mouse (Gardner *et al.* 1935). Burrows (1935) reports that equilin administration caused alveolar proliferation in the mammae of male mice. When the oestrogen dosage is high and the treatment prolonged, neoplasms are formed in the mammary gland of the mouse, particularly in cancer-susceptible strains (for references see Gardner, 1937), while high doses of oestrogen over short periods stimulate a stunted type of duct development (Gardner *et al.* 1936; Van Heuverswyn *et al.* 1939). Oestrogen dosage and age when administered were shown by Astwood *et al.* (1937) to influence the type of response of the mammary gland in the rat.

In most species it has been found that progesterone alone causes no mammary development, though complete development of duct and alveolar systems results from simultaneous treatment with oestrogens and progesterone. The male mouse appears to be an exception, since Gardner & Hill (1936) reported duct growth in mice following treatment with progesterone. It is interesting in this connexion

[1] *Oestrogens* is the general name used in this review in respect of oestrus-producing hormones. Certain naturally occurring oestrogens belonging to the steroid group are referred to here under their common chemical names *oestrone*, *oestradiol*, *oestriol* and *equilin*. *Progesterone* is the progestational hormone of the corpus luteum. *Androgens* are substances which promote growth of the accessory reproductive organs in castrated males. *Testosterone* and *cis-androsterone* are androgens which are found in the testis and male urine respectively.

that Van Heuverswyn *et al.* (1939) found that the adrenal cortical hormone, desoxycorticosterone, caused duct growth in the same species. It is well known that progesterone possesses adrenal-cortex hormone activity. The male goat also seems to be an exception to the above generalization though in the opposite sense to the mouse. Hitherto it has not been possible to stimulate mammary development in male goats even when progesterone is given in addition to oestrogen (Folley *et al.* 1940).

In concluding this section it is perhaps not out of place to remark that controversies as to whether or not oestrogens alone will cause alveolar development in males and ovariectomized females have lost most of their point since the isolation of progesterone from the adrenal cortex by Beall & Reichstein (1938). Alveolar development produced by oestrogen administration in males and spayed females may be due to the combined action of oestrogen and progesterone from the adrenal cortex.

### (2) *The effect of androgens.*

Certain observations (see Bottomley & Folley, 1938 for discussion) point to a relation between the testes and the mammary gland in the male; it is therefore not surprising that development of the mammary gland has been experimentally produced by administration of certain androgenic substances. Selye *et al.* (1936) were the first to show that in the rat, mammary development is stimulated by administration of testosterone benzoate. Similar results with uncombined testosterone were obtained by Astwood *et al.* (1937). Nelson & Gallagher (1936) found that the semi-synthetic androgens "androstendion" and "androstandiol" were active in the rat while androsterone was inactive. Bottomley & Folley (1938) studied the action of a series of androgens on the teat and mammary gland of the young male guinea-pig and concluded that the presence in the molecule of a double bond ($\Delta_4$ or $\Delta_5$) and at least one hydroxyl group (in the 3 or 17 position) was favourable for teat-growth promotion. The most active substance studied was $\Delta_5$-*trans*-androstendiol, while $\Delta_4$-androstendione, *cis*-androstendiol, dihydrotestosterone and *cis*-androsterone had very little activity. In these experiments relatively little duct and alveolar growth was observed even with $\Delta_5$-*trans*-androstendiol. Androgenic stimulation of nipple growth in the guinea-pig has also been observed by Jadassohn *et al.* (1938).

Van Heuverswyn *et al.* (1939) observed considerable mammary duct growth in male mice following androgenic stimulation. Testosterone and $\Delta_4$-androstendione were the most active, and *cis*-androsterone the least active of the androgens studied.

The effect of androgens on the mammary gland of the ovariectomized virgin female rhesus monkey has been studied by van Wagenen & Folley (1939). Testosterone propionate, $\Delta_5$-*trans*-dehydro*iso*androsterone, $\Delta_5$-*trans*-androstendiol and *cis*-androsterone all failed to stimulate duct growth, though testosterone propionate caused alveolar growth in glands containing pre-existing alveoli. Testosterone propionate, and to a lesser degree the other unsaturated members of the series,

also caused duct dilation, due to induction of secretory activity a phenomenon similar to that observed in the rat after testosterone propionate treatment by Reece & Mixner (1939). Testosterone and its propionate also cause an abnormal puckering of the mammary epithelium in the monkey (Folley *et al.* 1939; van Wagenen & Folley, 1939).

### (3) *The effect of the anterior pituitary*

The question arises whether the ovarian hormones promote mammary growth by direct action on the mammary gland or whether their action is mediated by the pituitary. The results of Corner (1930), who observed mammary alveolar development (and lactation) in ovariectomized rabbits which had never ovulated, following injections of anterior lobe extracts, suggested that the pituitary might produce hormones capable of causing mammary growth. Unfortunately the results of experiments on the effect of ovarian hormones on the mammary glands in hypophysectomized animals have been conflicting, so that the present position is difficult to assess.

On the one hand, lack of mammary development after oestrogen treatment (in some cases in combination with progesterone) in hypophysectomized animals was reported in the rat by Reece *et al.* (1936), and in the mouse, cat, guinea-pig and ground squirrel (*Tamias striatus*) by Gomez & Turner (1937*a*). Further, McEuen *et al.* (1937) observed no mammary development in hypophysectomized rats following testosterone treatment, though testosterone promotes development in the intact animal (see above). In the hypophysectomized guinea-pig, it is interesting to note, oestrogen promoted nipple growth (Gomez & Turner, 1936*a*, 1937*a*; Lyons & Pencharz, 1936), indicating that the mechanism of nipple growth is distinct from that governing the growth of the mammary ducts and alveoli. Lyons & Pencharz (1936) observed some duct and alveolar development in oestrogen-treated hypophysectomized guinea-pigs but much less than in controls.

On the other hand, oestrogens (sometimes with progesterone) have been reported to promote mammary growth after hypophysectomy in the rat by Ruinen (1932) and Freud & de Jongh (1935), guinea-pig by Nelson (1935*a*), rabbit by Asdell & Seidenstein (1935) (see also Asdell *et al.* 1936) and dog by Houssay (1935*a*).

The need for care in ensuring complete hypophysectomy is emphasized by the work of Gomez, Turner, Gardner & Hill (1937), who found that though oestrogens stimulated no mammary development in completely hypophysectomized mice, development occurred if even microscopic pituitary fragments remained in the *sellae*. It may be noted, however, that Asdell *et al.* (1936) based their conclusions on four rabbits which had been completely hypophysectomized as judged by microscopic examination of serial sections of the *sellae*.

Gomez, Turner & Reece (1937) (see also Gomez & Turner, 1937*a*) claim to have obtained positive evidence that the path by which ovarian hormones stimulate growth of the mammary ducts and alveoli is indirect and indeed by way of the

pituitary, since mammary development was stimulated in completely hypophysectomized castrated guinea-pigs by daily implantation of pituitaries from donor rats which had been injected with oestrogens, while implantation of pituitaries from control rats had no such effect.

Later, Gomez & Turner (1938) claimed to have obtained further evidence for the existence of a pituitary "mammogenic" hormone, because extracts of anterior lobes from cows killed in pregnancy, when the blood oestrogen is high, stimulated mammary growth in rabbits and rats, material from non-pregnant cows proving inactive. The "mammogenic" hormone is reported to be soluble in fat solvents (Lewis & Turner, 1938) and a method for its assay, using male mice, has been developed (Lewis *et al.* 1939). Reece & Leonard (1939) support the existence of a pituitary "mammogenic" factor, since they also observed mammary growth in hypophysectomized rats which received daily implants of pituitaries from oestrogen treated "donors". Pituitaries from untreated "donors" were equally effective in hypophysectomized rats, be it noted.

Nelson (1938) was unable to obtain evidence of the existence of a "mammogenic" hormone since he found that implants of pituitaries from normal rats were equally, if not more, effective than those of oestrogen-treated "donors" in growing the mammae of hypophysectomized recipients. Later (1939*a*), he found that pituitaries from oestrogen-treated "donors" caused no mammary growth in female rats or male mice. Astwood *et al.* (1937) confirmed the lack of effect of oestrogens on the mammary gland in hypophysectomized rats, but showed that this might be due to the lowered plane of nutrition following hypophysectomy. Nathanson *et al.* (1939), who observed partial mammary growth in hypophysectomized rats given oestrogen together with anterior lobe extracts, also consider that nutrition is an important factor, but believe that oestrogen may need some co-operation from the pituitary in promoting mammary growth. Nelson & Tobin (1936–7) observed the mammary growth-promoting action of oestrogen in hypophysectomized rats receiving in addition crude pituitary extracts, but opposite results were reported by Selye & Collip (1936). Lewis & Turner (1939) explain this by assuming that the anterior lobe extracts used by Nelson & Tobin contained "mammogenic" hormone while those of Selye & Collip did not.

Gomez & Turner (1937*a*) are inclined to exclude the alternative possibility that mammary development is due to the synergistic action of ovarian and pituitary hormones, since the administration of ovarian hormones to hypophysectomized rats and guinea-pigs in combination with various pituitary preparations failed to stimulate mammary growth (but see Nathanson *et al.* 1939).

In view of the demonstration by Van Heuverswyn *et al.* (1939) of the mammary growth-promoting action of an adrenal cortex hormone in the male mouse, the possibility that the effects ascribed to an anterior lobe "mammogenic" hormone might be due to a substance which stimulates the adrenal cortex must be considered. In any event, though the work of Turner and his school is of the greatest interest and may open up an entirely new field, wider confirmation is desirable before the existence of pituitary hormones which stimulate mammary growth can be accepted.

## II. ENDOCRINE FACTORS IN LACTATION

### (1) *The role of the anterior pituitary in lactation*

An impressive array of experimental evidence has accumulated within recent years indicating that the anterior pituitary plays a major role in milk secretion. In numerous species it has been shown that hypophysectomy during pregnancy will prevent normal lactation, even though live young are born. Similarly, hypophysectomy performed during lactation invariably causes complete and rapid cessation of milk secretion. Many experiments in support of these statements are quoted by Nelson (1936), Folley (1938*a*) and Turner (1939). In many cases, parturition in hypophysectomized animals appears to be followed by milk secretion for a short time (see Pencharz & Lyons, 1934 for the guinea-pig and Selye *et al.* 1933*a*, *b* for the rat and mouse).

Further, it has been found that certain experimental procedures which normally bring about lactation in animals with intact pituitaries do not so result in hypophysectomized animals. Thus neither surgical removal of all foetuses from the rat in late pregnancy (Collip *et al.* 1933), nor removal from the non-pregnant rat of the ovaries intensely luteinized by injections of chorionic gonadotrophin (Selye *et al.* 1933*c*) result in lactation if the pituitary is simultaneously removed. Similarly, Nelson (1935*a*) found that removal of ovarian grafts from male guinea-pigs which have carried them for some time, or cessation of a course of injections of oestrone in the male or female guinea-pig, was not followed by lactation in hypophysectomized animals, though such treatment does produce lactation in animals with intact pituitaries. Similar results with male guinea-pigs were reported by Lyons & Pencharz (1936).

The experiments just considered unanimously point to the existence of a hormone or hormones of the pituitary, essential for the initiation and maintenance of lactation. Positive evidence of the existence of such hormones in the anterior pituitary was first obtained by Stricker & Grueter in 1928, who found that injection of aqueous anterior pituitary extracts into ovariectomized pseudo-pregnant rabbits initiated lactation. The ability of such extracts to initiate lactation in glands presumably possessing a certain amount of alveolar development was quickly confirmed in experiments on a variety of species (for citations see reviews by Nelson, 1936; Folley, 1938*a*; and Turner, 1939).

The existence of a specific anterior lobe lactogenic hormone is almost universally accepted at the present time and various names for it have been proposed, the most well known being prolactin (Riddle), galactin (Turner) and mammotropin (Lyons). The first is used here as it has been generally adopted in this country.

The most widely used methods of assay of the potency of preparations of prolactin are based on the interesting observation of Riddle *et al.* (1933) that anterior pituitary extracts which are lactogenic will also cause growth of the crop glands in the pigeon. Systemic assay methods involving intramuscular or subcutaneous injections and determinations of crop-gland weights have been described

by Riddle *et al.* (1933), Dyer (1936) and Rowlands (1937). McShan & Turner (1936) described a systemic minimal stimulation method, while Lyons (1937*a*) has devised a minimal stimulation method in which injections are made intradermally over the crop and by which very small amounts of prolactin can be detected. The use for assay purposes of the lactogenic response in the mammal, while for obvious reasons much more desirable, is fraught with greater difficulties, though Gardner & Turner (1933) proposed a method of assay based on the initiation of milk secretion in the pseudo-pregnant rabbit, and Nelson (1934) has advocated the use for this purpose of the guinea-pig hysterectomized during pregnancy. Methods of assay of prolactin have been discussed by Bates (1937), Lyons (1937*b*), and Riddle & Bates (1939).

Since there is no rigid proof that the substance which causes growth of the pigeon crop does possess lactogenic properties because no pure preparation of the crop-stimulating factor has yet been made, it seems desirable to use the name prolactin in respect of crop-stimulating activity only, without prejudice to the question whether or not all prolactin preparations have the power of stimulating the production of *normal* milk in mammals.

Considerable progress has been made in obtaining prolactin preparations which are largely free from other anterior lobe hormones. For this purpose Bates & Riddle (1935) recommend extraction of anterior lobe tissue with 70% alcohol at *p*H 9–10 followed by precipitation at *p*H 6. Subsequent repeated re-solution at *p*H 9 followed by precipitation at *p*H 3–4 removes practically all gonadotrophic and thyrotrophic hormones. Other procedures for obtaining potent prolactin preparations have been described by Lyons & Catchpole (1933*a*) and McShan & Turner (1935). Lyons (1937*a*) has developed a method of preparation involving extraction with acid acetone which, he claims, allows of a reasonably good separation of the crop-stimulating activity from the anterior pituitary adrenotrophic factor.

Comparatively little is known at present about the chemical nature of prolactin though the loss of activity on proteolysis (Bates *et al.* 1934; McShan & French, 1937), its solubility properties (see Riddle & Bates, 1939) and the preparation of an anti-prolactin serum (Young, 1938*a*) would indicate that it is a protein. Its activity appears to depend on the presence in the molecule of free amino groups since inactivation follows acetylation with ketene (Li, Simpson & Evans, 1939), treatment with nitrous acid (Li, Lyons, Simpson & Evans, 1939) and treatment with phenyl *iso*cyanate (Bottomley & Folley, 1940). The announcement by White *et al.* (1937) of the preparation of a crystalline protein possessing crop-stimulating activity is of great interest, and confirmation of this together with reports on the chemical and physiological properties of this substance, which may prove to be the first anterior pituitary hormone to be isolated in the crystalline condition, are awaited. One such report (Shipley *et al.* 1939) indicates that solutions of "crystalline" prolactin are electrophoretically homogenous as are solutions of Lyons prolactin (Li, Lyons & Evans, 1939).

The anterior lobe of the pituitary is the chief source of prolactin. It appears to be mainly produced by the eosinophils (Azimov & Altman, 1938). The prolactin

content of the anterior pituitary in cattle of both sexes and various ages has been studied by Bates *et al.* (1935) and of the pituitary in various species by Reece & Turner (1937*a*). Prolactin has also been detected in the urine of lactating women (Lyons & Page, 1935), babies secreting "witches' milk" (Lyons, 1937*c*) and normal human males (Lyons, 1937*b*) and in the blood serum of mares (Leblond, 1937).

Whether or not administration of anterior lobe extracts containing prolactin to hypophysectomized animals will initiate and maintain lactation in circumstances under which lactation would proceed in the intact animal is a point of great importance. The experimental evidence bearing upon this has until recently been somewhat conflicting. Removal of the pituitary disturbs a number of endocrine relationships, the normal functioning of many if not all of which is necessary for the maintenance of lactation (see below). It is, therefore, not surprising that partially purified anterior lobe extracts may fail to evoke lactation in hypophysectomized animals, although they are potent as regards the initiation and maintenance of lactation in intact animals possessing the necessary degree of mammary development.

The experiments of McPhail on the cat (1935*a*) and ferret (1935*b*), of Houssay (1935*b*) and of Lyons *et al.* (1933) on the dog, and of Riddle *et al.* (1933) on the rat, indicated that relatively crude extracts of anterior lobe (in the latter case a partially purified preparation of prolactin was used) will initiate and support lactation in the hypophysectomized animal possessing the requisite mammary development. On the other hand, Selye *et al.* (1934) could not maintain lactation in hypophysectomized rats with daily implants of pituitaries, nor could Nelson (1936) maintain lactation in rats after hypophysectomy, by injection either of crude anterior lobe extracts or of purified prolactin preparations. It is probable that these somewhat conflicting results were due to differences in the hormone content of the extracts used.

Evidence which supports this explanation has since come to hand. Nelson & Gaunt (1936) found that while crude anterior lobe extracts would initiate lactation in guinea-pigs which had been prepared for lactation by treatment with oestrogenic hormones and hypophysectomized just before the injections were begun, partially purified prolactin preparations had no such effect. Similar results were reported by Gomez & Turner (1936*b*), who found that partially purified prolactin would not initiate, re-initiate, or prevent the cessation of lactation in hypophysectomized guinea-pigs. These findings indicated that the failure of the partially purified prolactin preparations to initiate lactation in the hypophysectomized animal was due to the loss of one or more anterior pituitary hormones during purification. Adrenal insufficiency, due to absence of the adrenotrophic hormone from the purified prolactin preparations, rather than hypothyroidism, due to the loss of the thyrotrophic hormone, seemed to be the cause of the failure of such preparations to support lactation, because: (*a*) administration of thyroxine together with prolactin failed to initiate lactation in the hypophysectomized animal (Gomez & Turner, 1937*a*, *c*), and (*b*) Nelson & Gaunt (1936) and Gomez & Turner (1936*c*, 1937*a*) found that lactation was initiated in hypophysectomized animals if adrenocortical

extract was administered together with the purified prolactin. Gomez & Turner (1937*a*, *b*) further showed that lactation could be re-initiated, and under favourable conditions maintained, in hypophysectomized guinea-pigs by administration of purified prolactin and glucose together with a preparation of adrenotrophic hormone (see also Nelson & Gaunt, 1937*a*, *b*, who obtained similar results).

The failure of partially purified crop-stimulating extracts to evoke or maintain lactation in the hypophysectomized animal may be cited in support of the view which has been put forward by Folley & Young (1938, 1939) that the crop-stimulating hormone (prolactin) is not the sole "lactogenic" hormone produced by the anterior pituitary.

In proceeding to the consideration of the effect on established lactation of anterior pituitary extracts containing prolactin, it should be noted that most of the experiments to be cited have been carried out with relatively crude extracts containing other pituitary principles in addition to prolactin. There is a considerable amount of evidence, obtained exclusively from experiments on larger animals, that administration of anterior pituitary extracts will stimulate lactation at any rate during the period of gradual decline. Experiments on the cow will be considered first as these have hitherto given most information.

Grüter & Stricker (1929) caused stimulation of lactation in cows by injections of anterior pituitary extracts, while Stockklausner & Daum (1932) found that such injections diminished for a time the normal rate of decline of lactation. Azimov and his collaborators (Azimov *et al.* 1933; Azimov *et al.* 1936; Azimov & Krouze, 1937) brought about temporary increases in milk yield by single and also repeated injections of alkaline extracts of anterior pituitary into cows. According to the last-named paper, which described experiments on 510 cows and ninety controls, the injections appeared to be most effective in early lactation. Indeed, evidence was obtained that anterior pituitary injections given at the peak of the lactation curve will increase the milk yield above the normal maximum. Evans (1936) was able temporarily to increase the milk yield of low-yielding cows by injections of a partially purified prolactin preparation.

Folley & Young (1937, 1938) have shown that single injections of crude anterior lobe extracts as well as partially purified preparations containing prolactin will temporarily increase the milk yields of cows in declining lactation, as will also repeated daily injections of prolactin made by Young's (1938*b*) modification of the method of Bates & Riddle (1935). They also found that a fraction exhibiting thyrotrophic and diabetogenic activity, but no crop-stimulating activity, stimulated bovine lactation in single injections. Among the extracts used, a good correlation existed between the glycotropic ("anti-insulin") (Young, 1936), and the lactogenic activities (single injection), while the correlation between the latter and the crop-stimulating activities was rather poor. In view of these results, it seems possible that prolactin may not be a specific lactogenic hormone and that the lactogenic properties of prolactin preparations may be due at least in part to the presence of other anterior lobe hormones.

Of great importance was the fact that in the one (albeit well-controlled) experi-

ment in which repeated high doses (of the order of 4000 I.U. daily) of partially purified prolactin were administered, the lactation curve of the cow was undoubtedly displaced upwards over the remainder of the lactation period. This might be due to the presence in the extract of a substance which caused the growth of new mammary tissue (see also Corner, 1930 and Lyons & Catchpole, 1933 *b*).

Studying the effects of *repeated* injections of prolactin extracts, as well as extracts free from prolactin but containing thyrotrophin, into groups of lactating cows, Folley & Young (1939) observed marked increases in yield, particularly with the prolactin preparations, accompanied in the latter case by a great increase in milk fat secretion. The milk yield, during prolonged injections of a prolactin extract rose sharply and then gradually declined despite continuance of treatment. Consideration of the biological properties of the extracts used indicated that the effects on milk secretion could not be ascribed to the action of a single hormone.

In the goat stimulation of established lactation by injections of anterior lobe extracts was reported by Grueter (1930). Asdell *et al.* (1936) succeeded in temporarily increasing the rate of milk secretion in goats by anterior pituitary injections during the latter stages of lactation, but the injections were ineffective at the peak of lactation, these findings with the goat differing in this respect from those of Azimov & Krouze (1937) with the cow. Stimulation of lactation with anterior pituitary extracts in the ewe has been accomplished by Kabak & Kisilstein (1934) and Kabak & Margulis (1935). In man, Kurzrok *et al.* (1934) and Kenny & King (1939) claim some success in the treatment of subnormal lactation by injections of prolactin. On the other hand, disappointing results were reported by Stewart & Pratt (1939), while possible dangers attending prolactin treatment in human beings are illustrated by the report of Werner (1939).

At the present time it is impossible to say whether or not prolactin itself is capable of increasing the rate of established lactation. All of the prolactin preparations used hitherto seem to have contained additional anterior pituitary factors, and the work of Folley & Young (1938, 1939) indicates that crude anterior lobe extracts contain at least one factor other than prolactin which will enhance established milk secretion.

### (2) *The initiation of lactation*

Prior to the realization of the lactogenic function of the pituitary it was customary to regard lactation as a process of passive nature, the onset of which at parturition followed the removal of the stimuli causing the gestational growth of the mammary glands. The idea that the onset of lactation is due to the removal of an inhibition appears to have been first entertained by Hildebrandt (1904), and the corpus luteum was at one time regarded as the source of such an inhibitor. The work of Hammond (1917) and Drummond-Robinson & Asdell (1926) appeared to lend support to this idea. The latter workers showed that in the pregnant goat ablation of the corpora lutea, sufficiently late in pregnancy for development of the alveolar system of the mammary gland to have occurred, resulted in immediate and copious lactation. Anselmino & Hoffman (1936), while inclining to the view that the corpus luteum does produce a substance which inhibits lactation, held that this substance

must be different from the progestational hormone, since concentrates of the latter failed to inhibit lactation in the rat. Later, Folley & Kon (1937) clearly demonstrated that injections of crystalline progesterone do not inhibit established lactation in the rat.

The power of oestrogenic hormones to inhibit lactation is, on the other hand, well established. The experiments of Laqueur *et al.* (1928), de Jongh & Dingemanse (1931), Steinach *et al.* (1928), Nelson (1935*b*) and others have demonstrated that in the guinea-pig removal of the stimulus to mammary growth provided by oestrogenic hormones results in immediate lactation. Further, it has repeatedly been shown that administration of oestrogenic or gonadotrophic hormones to lactating animals results in inhibition of lactation. Such experiments have been cited by Folley (1936) and Turner (1939). The clinical use of oestrogens for suppressing lactation in women has been reported by Ramos & Colombo (1938), Foss & Phillips (1938), Lehmann (1938), Winterton & MacGregor (1939) and Kellar & Sutherland (1939).

Incidentally, it has recently been shown that certain androgens which possess the power of stimulating mammary growth will also inhibit lactation. Thus Folley & Kon (1937) find that testosterone but not *cis*-androsterone will inhibit lactation in the rat. Testosterone, as we have seen, promotes mammary growth in rats, mice and guinea-pigs, but *cis*-androsterone has little effect. There is some evidence therefore that the power of promoting mammary duct growth goes hand in hand with the ability to inhibit lactation.

It must be admitted that the amounts of oestrogen necessary to inhibit lactation experimentally are much higher than are likely to operate in the pregnant animal, so that it is possible that some additional inhibitory influence is operative during pregnancy (see below).

Anselmino & Hoffman (1936) claim that the lactation inhibitory effect of oestrogenic hormones in the rat can only be demonstrated in the presence of the ovary, and they suggest therefore that the real inhibitor of lactation may be a substance produced by the corpora lutea, the formation of which is promoted by oestrogenic hormone treatment. On the other hand, de Jongh (1933) and Robson (1935) both succeeded in demonstrating the inhibitory effect upon lactation of oestrogenic hormones in the ovariectomized mouse. The dosage of oestrogenic hormone employed by Anselmino & Hoffmann would appear to have been insufficient to inhibit lactation in the absence of the ovary, since Folley & Kon (1937) found that though oestradiol monobenzoate would inhibit lactation in ovariectomized rats, the degree of inhibition was less than that caused by equal doses in intact females.

The discovery of the positive lactation stimulus provided by the pituitary has necessitated the inclusion of this factor in any theory attempting to explain the mechanism of the initiation of lactation. Nelson (1936) has advanced a theory according to which oestrogenic hormones, probably of placental origin, inhibit lactation during pregnancy (*a*) by suppressing the secretion of lactogenic hormone by the hypophysis, and (*b*) by a direct action on the mammary glands. The removal

of this inhibition consequent upon the decrease of oestrogens present in the blood, which apparently occurs at parturition (see Ascheim & Zondek, 1927; Nibler & Turner, 1929), permits the release of prolactin from the hypophysis and the consequent initiation of lactation. Nelson has put forward a considerable amount of experimental evidence in support of this theory, which is also supported by the aforementioned experiments on the inhibition of lactation by oestrogenic hormones, and by the experiments referred to in a preceding section which show that certain procedures which initiate lactation in the intact animal fail to do so in absence of the hypophysis. The lactation of a few hours' duration which was observed by Selye *et al.* (1933 *a*, *b*) in rats and mice hypophysectomized before parturition is difficult to explain on the basis of this theory, unless one accepts the conclusion of these authors that the pregnant uterus and its contents is, under certain circumstances, capable of producing a substance with the physiological activity of the anterior lobe lactogenic factor. A further difficulty is the failure of Selye *et al.* (1934) to cause inhibition of lactation in lactating mice by daily implantations of whole mouse placentae, though, on the other hand, Frankl (1923) was able to inhibit milk secretion in the mouse by placental grafts.

Reece & Turner (1937 *a*, *b*) believe that oestrogens do not suppress the secretion of lactogen by the pituitary during pregnancy, since they found that the pituitary of the pregnant rat contains less prolactin than that of the oestrus female, while injection of oestrogens into the spayed female increases the prolactin content of the pituitary, ovariectomy having the reverse effect. It may be that oestrogens inhibit lactation by directly decreasing the response of the mammary gland to the lactogenic hormone, since oestrogen administration will markedly diminish the response of the pigeon crop-gland to prolactin (Folley & White, 1937; Folley & Scott Watson, 1938; Folley, 1939) though such reasoning assumes the identity of the lactogenic and crop-stimulating factors.

Other results which up to the present have not been satisfactorily explained on existing theories point to the intervention of a uterine factor in the chain of events leading to normal lactation. Selye *et al.* (1934) showed that on the one hand removal of the foetuses from the pregnant rat by Caesarian section induced lactation, but on the other no lactation resulted if the uterus was immediately distended with paraffin. These authors urge that the mechanical distension of the uterus must be considered as a factor causing the inhibition of the secretory phase during gestation, and point out that the distension of the uterine walls decreases just before parturition despite the continued growth of the foetuses. The position is however complicated by the results of Freud & Wijsenbeek (1938). Rat foetuses were transferred to the abdomen but no lactation occurred until they were artificially delivered, from which it would appear that factors other than uterine distension must be considered. A relation between the uterus and lactation is also indicated by the findings of Bradbury (1932). He showed that hysterectomy will prevent the formation of interlobular canals in the mouse mammary gland, even though functional corpora lutea are present in the ovaries, while hysterectomy was followed by milk secretion in mice possessing mammary alveolar development.

### (3) *The adrenal cortex and lactation*

The possibility of the existence of a relationship between the adrenal cortex and lactation has been emphasized by comparatively recent work. The studies of Carr (1931), Swingle & Pfiffner (1932), Gaunt (1933), Britton & Kline (1936), and others, indicate that adrenalectomized animals which for one reason or another survive over the experimental period are incapable of normal lactation. It may therefore be concluded that the integrity of the adrenal cortex is essential for normal lactation, a conclusion supported by the inability of partially purified prolactin preparations to initiate or re-initiate lactation in animals hypophysectomized during pregnancy or lactation respectively, unless adrenotrophic or adrenal cortex hormones are administered simultaneously (see citations above).

The position, which until recently was somewhat obscure owing to disagreement between various workers as to whether or not adrenalectomized animals can lactate if life-maintaining doses of adrenal cortex hormone are administered, has been clarified by the work of Gaunt & Tobin (1936) which emphasizes the importance of dosage. These authors found that adrenalectomized rats treated with cortical hormone at a level just sufficient to maintain life, lactated poorly. Administration of salt gave slightly better results, but lactation was only normal when cortical hormone was injected at a relatively high level. Anterior lobe lactogenic preparations failed to improve the subnormal lactation on lower dosages of cortical hormone.

These results do not necessarily support the view put forward by Brownell *et al.* (1933) that the adrenal cortex secretes a specific lactation hormone. On the basis of present knowledge it is perhaps more reasonable to suppose that the relation between the adrenal cortex and lactation is indirect, and that the failure of lactation in animals suffering from experimental adrenal insufficiency is due to the inability of the adrenalectomized animals to meet the heavy demands of lactation upon the general metabolism. (For further discussion see Nelson & Gaunt, 1937*b*).

Another aspect of the connexion between the mammary gland and the adrenal cortex has been opened up by the demonstration by Van Heuverswyn *et al.* (1939) that desoxycorticosterone, a substance known to be present in the adrenal cortex, will cause mammary development in the mouse.

### (4) *The thyroid gland and lactation*

Since the thyroid gland is a regulator of metabolism it is to be expected that its internal secretion would exert an influence on lactation. There are at least three possibilities, first that the thyroid hormone may govern the level of milk precursors in the blood (see Jones, 1935), secondly that it may affect the rate of blood flow through the mammary gland, and indeed Folley & White (1936) observed an increase in pulse rate in cows receiving thyroxine injections, while Fuller (1928) reported a positive correlation between pulse rate and milk yield, and thirdly it may directly govern the metabolic rate of the mammary gland cells.

Let us first deal with studies on lactation in thyroidectomized animals. Trautmann (1919) observed a decrease in milk yield after thyroidectomy in goats. Similar

results by Grimmer (1918) were ascribed to operative disturbance since the decline was but temporary. Graham (1934*a*) observed a decrease in milk yield following thyroidectomy in the cow, but the result cannot be regarded as clear-cut since a control operation had a somewhat similar result. Apparently normal lactation following thyro-parathyroidectomy in bitches was reported by Dragstedt *et al.* (1924) provided tetany was controlled.

Nelson & Tobin (1937) state that they could obtain no evidence that if rats were thyro-parathyroidectomized in pregnancy, milk secretion was depressed during the subsequent lactation. Folley (1938*b*), on the other hand, found that in lactating rats thyro-parathyroidectomy immediately exerted an adverse effect on milk secretion. If the operated rats were remated the subsequent lactation was definitely subnormal, indicating that in the rat thyro-parathyroidectomy does seriously interfere with lactation. Nelson (1939*b*) in a subsequent reinvestigation for the problem has reaffirmed the results of Nelson & Tobin (1937), and the reasons of the discrepancy between the results of the two laboratories are still to seek.

The above observations on the effect of thyroidectomy on lactation are somewhat conflicting, possibly partly because of species differences, but the general conclusion seems justified that in many species lactation can proceed in absence of the thyroid, but only to a slight degree.

The influence of the thyroid gland on lactation has been clearly established by administration of thyroid hormone to pregnant and lactating animals. Weichert & Boyd (1934) observed that in the pregnant rat experimental hyperthyroidism caused earlier mammary development and initiation of the secretory phase than in controls. Working with the lactating cow in declining lactation, Graham found that thyroid feeding (1934*a*) or thyroxine injections (1934*b*) caused a considerable temporary increase in milk fat yield. Such treatment appeared to be much less effective in the early stages of lactation.

Folley & White (1936), studying a group of cows receiving thyroxine injections, confirmed Graham's results and further found that the percentage and yield of milk non-fatty solids were increased by the treatment, in this respect differing from Jack & Bechdel (1935). Similar results were later obtained by Herman *et al.* (1938) who, however, observed only a relatively slight increase in non-fatty solids secretion. Contrary results by de Fremery (1936) with the goat were probably due to overdosage with thyroxine. Folley & White (1936) observed that thyroxine treatment raises the milk yield to a higher level, from which it declines at the normal rate, even though the treatment is continued. The above work suggests that the thyroid gland is intimately concerned in milk-fat secretion in particular, since in experimental hyperthyroidism the fat content of cow's milk increases much more than that of the non-fatty constituents.

In the cow, Folley & Young (1938, 1939) found that, as would be expected, injections of extracts containing anterior lobe thyrotrophin stimulate milk secretion, though it must be noted that thyrotrophic extracts did not increase the milk fat content nor was the relation between the lactogenic and thyrotrophic activities of the extracts very close. In the guinea-pig, thyrotrophic hormone caused, on the

contrary, a diminution in lactation (Grumbrecht & von Düsterlho, 1937), which was partially rectified by administration of di-iodotyrosin. It is of interest to note that Küstner (1934) claimed that administration of di-iodotyrosin stimulated lactation in women who, from their previous histories, might be expected to lactate subnormally.

### (5) *The posterior pituitary and lactation*

Ott & Scott (1910) were probably the first to show that injections of extracts of posterior pituitary produces an increase in the rate of flow of milk from the lactating mammary gland. They found that a single injection into a lactating goat resulted in an increase in the rate of flow of milk from a cannula inserted into the udder. The effect was temporary, lasting in their experiments for a few minutes only. Of the two possibilities, namely, (*a*) that posterior pituitary extracts affect the rate of milk secretion through their action on the alveolar epithelium, and (*b*) that the rate of excretion of preformed milk from the mammary gland is increased by posterior lobe extracts, the first was supported by Simpson & Hill (1914–15).

Overwhelming evidence presented by other workers, however, has shown that the second explanation is correct. Schäfer (1913) and Hammond (1913) found that though injection of posterior lobe extracts undoubtedly causes an immediate and temporary increase in the rate of milk flow, there is little or no increase in yield over a long period, and Schäfer (1914–15) further showed that a second injection of posterior lobe extract soon after the emptying of the gland due to one injection, did not produce any milk. More recent work of Turner & Slaughter (1930) also indicates that posterior lobe extract is not a true galactogogue. In spite of Fauvet's (1932) contention that the posterior lobe secretes a lactogenic hormone, it is safe to conclude that posterior lobe extracts affect only the mechanism which governs the excretion of milk from the mammary gland. A significant fact to be noted in this connexion is the rapidity of the mammary response to injections of posterior lobe extract, in contrast to the time which elapses before any increase in milk yield due to injections of anterior lobe lactogenic extracts becomes evident. The above conclusion is supported by the results of Smith (1932) and Houssay (1935*c*) who showed in the rat and dog respectively that lactation is possible after ablation of the posterior lobe of the pituitary.

There remains the interesting possibility, as yet unsupported by any experimental evidence, that the posterior lobe of the pituitary gland may play a part in the discharge of milk from the mammary gland during normal suckling.

### (6) *Endocrine control of milk composition*

Elucidation of the mechanism controlling milk composition is a matter of great practical importance. In past studies of bovine lactation, attention has been mainly focused upon the fat content of milk, but the comparatively recent recognition of the tendency of many cows, particularly of high-milking breeds, to secrete milk containing less than is desirable of the non-fatty constituents (which are quite as important as the fat from the nutritional point of view), has emphasized the

necessity for research in this field. It is reasonable to assume that the hormones which have been found to control mammary development and the initiation of secretion may also participate in the control of milk composition. Such control probably operates in various ways, two of the most likely being (1) the control of the level of milk precursors in the blood, and (2) the control of the efficiency of synthesis of any or all of the milk constituents, given an adequate supply of precursors.

Since changes in milk composition occur after thyroidectomy, it may be inferred that the thyroid gland is concerned in the regulation of milk composition. Von Fellenberg & Grüter (1932) found that in the lactating goat, thyroidectomy lowers the calcium and phosphorus contents of the milk and increases the chloride content. Conversely, thyroid feeding or thyroxine injections in the cow in advanced lactation cause a very marked increase in milk fat content (Graham, 1934*a*; Folley & White, 1936; Herman *et al.* 1938) and an increase of smaller magnitude in the non-fatty solids content (Folley & White, 1936; Herman *et al.* 1938). According to Graham & Jones (quoted by Jones, 1935) the increase in non-fatty solids content is partly due to increased secretion of lactose. In this respect the findings of von Fellenberg & Grüter are in accord since the concentrations of chloride and lactose in milk are inversely related (Mathieu & Ferré, 1914). The above results strongly suggest that the thyroid gland participates in the regulation of the composition of the milk.

Folley (1936) (see also Folley & Scott Watson, 1938) has obtained evidence which points to the important role played by oestrogenic hormones in the control of milk composition. Administration of oestrogenic hormones to cows in declining lactation caused considerable increases in the percentages of milk fat and milk non-fatty solids, which moreover lasted for considerable periods of time. The concentrations of the various nitrogenous constituents of the milk rose, but the partition of nitrogen remained characteristic of normal milk (Davies, 1935) showing that treatment with oestrogenic hormones during lactation did not promote colostrum secretion, as de Fremery (1938) believes to happen in the goat. If the treatment had caused the secretion of colostrum, the percentage of the total milk nitrogen as globulin nitrogen would have risen considerably, but in fact this did not happen. Whether or not the interesting changes in milk composition which follow injections of oestrogenic hormones are connected with the power of these substances to stimulate mammary growth or whether they are connected with the water-retaining properties of oestrogens (see Zuckerman *et al.* 1939), remains for future work to decide.

Folley & Young (1938) have studied the effect of injections of anterior pituitary extracts on the composition of bovine milk. Single injections of various extracts into lactating cows were followed by only temporary and relatively minor alterations in milk composition, though evidence was obtained that repeated daily injections of a prolactin preparation caused a rise in the milk lactose accompanied by a fall in the chlorides. Repeated injections of certain anterior lobe extracts containing prolactin, however, caused a striking increase in the milk fat percentage (Folley & Young, 1939).

The effect of various hormone treatments upon the phosphatase (phosphomonoesterase $A_1$, Folley & Kay, 1936*a*) of cow's milk is of considerable interest. Thyroxine treatment causes a sharp temporary decline in milk phosphatase content (Folley & White, 1936) while administration of oestrogen causes an equally striking increase (Folley, 1936). In each case the change in enzyme concentration is correlated with a change in milk yield in the opposite sense. These phenomena, taken in conjunction with the typical lactation curve for phosphatase in cow's milk obtained by Folley & Kay (1936*b*), give rise to the conception that the phosphatase of milk which, as we shall see later, appears to originate in the mammary gland itself, is part of the equipment of the mammary gland cell necessary for its secretory function, that a high concentration of phosphatase in milk signifies a low level of mammary efficiency and that a sudden change in the concentration of this enzyme in milk may be taken as a sensitive indication of some change in the secretory efficiency of the mammary gland. It must be admitted, however, that it is difficult to explain why no appreciable and regular changes in milk phosphatase content accompanied the increase in milk yield resulting from injections of anterior lobe extracts in the cow (Folley & Young, 1938).

## III. NERVOUS INFLUENCES IN LACTATION

Turner (1932) should be consulted for an account of early work which showed that the mammary gland is not innervated by true secretory nerve fibres. There is a certain amount of evidence, however, that the sympathetic nervous system may have some influence on lactation. Basch (1906), who quotes references to early work, found that extirpation of the coeliac sympathetic ganglion had little effect on milk secretion. More recently, however, Bacq (1932) reported that abdominal sympathectomy in the rat occasionally interfered with lactation and Cannon & Bright (1931) observed that lactation in the cat was adversely affected by sympathectomy, though the effect was delayed until some time after the operation. In the experiments of Simeone & Ross (1938) lactation was impaired in only a few of a number of sympathectomized cats. The impairment was noticed in the second but not the first lactation after the operation.

The possibility should not be forgotten that the rate of milk secretion and also possibly milk composition might be to some extent dependent on sympathetic vasomotor impulses controlling the blood supply, and hence the supply of milk precursors and metabolites, to the mammary gland.

The intervention of nervous impulses in the actual act of milking or "letting down" of milk has been recently postulated by Hammond (1936), who has brought together a considerable amount of evidence, existing in the literature, in favour of his theory that the "letting down" of milk is an active nervous reflex excited by stimulation of the teat. Tgetgel (1926) has shown that there is a steady increase in pressure between one milking and the next, caused by the gradual accumulation of milk in the udder. The beginning of milking is, however, accompanied by a sudden increase in pressure, followed by a gradual fall as milking proceeds. Some workers

have attempted to account for the abrupt increase in milk pressure at the beginning of milking, as being due to reflex secretion of milk resulting from stimulation of the teats during the act of milking. This theory postulates that a considerable proportion of the milk obtained at any one milking must be secreted during the milking process, which on the face of it seems very unlikely and against the probability of which Hammond has adduced a considerable amount of evidence. For full discussion of this matter Hammond's paper must be consulted; it will suffice to quote here only the most telling evidence. Gaines & Sanmann (1927) and also Gowen & Tobey (1928), on the basis of determinations of the amount of lactose present in udders obtained from cows slaughtered at the normal milking time, have concluded that there was actually more milk present in such udders than could be withdrawn at corresponding milkings on previous days.

Hammond considers that the sudden rise in milk pressure at the commencement of milking can be best explained as due to reflex erection of udder and teat tissues caused by stimulation of the teats, the mechanism resembling that involved in erection of the penis. Afferent nerves conduct the impulses to a centre situated in the spinal cord, from which efferent impulses go to nerve endings in smooth muscle fibres associated with the venous system of the udder, the resulting erection causing occlusion of the vessels and accumulation of blood in the udder tissues, which in turn causes a rise in milk pressure. In Hammond's view this rise in milk pressure is essential for the "complete" emptying of the udder (that is as completely as is ever possible at one milking), and indeed it is difficult otherwise to account for the release of milk from the alveoli and the numerous ducts of small calibre, where the forces of capillary attraction must be of considerable magnitude. In support of this theory Hammond points out that the bovine udder contains a dense network of anastomosing blood vessels which, according to Nelke (1909), is capable of holding about one-half of the blood in the body. Further, much less milk can be obtained by catheterization of the udder, which involves no stimulation of the teats, than by milking in the normal way (Zwart, 1911; Dyssegaard, 1923), while experiments on the post-mortem milking of bovine udders amputated just before the normal milking time (e.g. Swett, 1927; Swett *et al.* 1932) have shown that under the most favourable conditions only about 70% of the milk obtainable at previous milkings can be withdrawn. Petersen *et al.* (1929*a*), on the other hand, obtained almost as much, and in some cases more, milk at post-mortem milkings of one half of an amputated bovine udder than they obtained by *in vivo* milking of the other half prior to slaughter. Hammond explains these results as follows. The erection of the udder caused by the *in vivo* milking prior to slaughter would force the milk present in the alveoli and finer ducts down into the larger ducts and cistern, from which it could be withdrawn by post-mortem milking. There is therefore considerable evidence that the "letting down" of milk is a nervous reflex actuated by stimulation of nerve endings in the teat.

Even though there appear to be no true secretory nerves to the mammary gland, so that the terminal (centrifugal) stage of the mechanism which evokes the secretion of milk in properly developed mammae is hormonal in nature, some interesting

experiments performed by Selye and his collaborators suggest, however, that nervous factors may be involved in the centripetal portion of the arc. Selye (1934) showed that if the period of suckling in the lactating rat is extended beyond the normal weaning time by the continued provision of actively suckling litters from other mothers, the mammary glands can be maintained in an actively secreting condition for long periods even if the escape of milk from the glands is prevented by cutting the galactophorous sinuses. Contrary to the results of Kuramitsu & Loeb (1921) and Hammond & Marshall (1925), it was found that if the suckling stimulus was applied to some of the nipples, secretion was maintained in the remaining glands where suckling had been prevented by removal of the nipples. Later, Selye & McKeown (1934*a*) found that application of the suckling stimulus to non-pregnant rats and mice caused the mammary glands to develop, while at the same time the normal succession of oestrus cycles was interrupted by dioestrus periods lasting from 2–3 weeks. The condition resulting from the suckling stimulus was named "suckling pseudo-pregnancy".

Selye and co-workers (for full discussion see Selye *et al.* 1934) interpret these experiments as showing that the suckling stimulus excites a nervous reflex which causes the secretion of lactogenic hormone by the anterior lobe, and in this connexion it is significant that injections of prolactin produce dioestrous periods in adult rats (Lahr & Riddle, 1936). Further evidence which suggests that lactation is maintained by impulses to the anterior lobe passing through the pituitary stalk has recently come to hand. Herold (1939) found that the sucklings of rats which underwent stalk transection died from starvation.

Selye and co-workers suggest that the involution of the mammary gland which follows weaning in experimental animals or gradual self-weaning of the young under natural conditions, is due to withdrawal of the suckling stimulus rather than to the destruction of the mammary gland cells due to the accumulation of milk under pressure. It is probable that in actual fact both causes contribute to the involution which follows weaning, though some other and as yet unknown cause may be operative, since in the case of such animals as the cow and goat it is well known that the milk yield gradually falls off after the peak of lactation has been reached in spite of the regular application of the milk stimulus to the teats and the regular emptying of the udder. Similarly, Selye & McKeown (1934*b*) found that in mice, though lactation may be prolonged for upwards of two months by the continual provision of actively suckling litters, mammary involution does occur eventually, although histologically it is of an abnormal type.

The application of tactile stimuli to the teat thus seems to affect the mammary gland in two ways. One mechanism is probably a purely nervous reflex which induces erection in the mammary gland tissues, while the other is partly nervous and partly hormonal in character. It is easy to see that while the first mechanism will act so rapidly as to account for the sudden rise in milk pressure at the beginning of milking, the effects of the other on milk secretion will probably take longer to evince themselves and may be expected to remain longer in evidence. Thus Folley & Young (1938) found that the stimulating effects on lactation in the cow due to

single injections of certain anterior pituitary extracts sometimes took two days to attain their full magnitudes and they disappeared comparatively slowly.

## IV. BIOCHEMICAL STUDIES ON MILK PRECURSORS

The importance, both from practical and theoretical standpoints, of elucidating the chemical reactions concerned in the synthesis of milk constituents in the living cell needs no emphasis. It is evident that a valuable contribution towards bridging the gap between blood and milk will have been made when it is known beyond doubt what blood constituents are used by the lactating mammary gland for the synthesis of each of the principal constituents of milk. The considerable effort which has been expended in attempting to determine the nature of milk precursors is, therefore, understandable. The nature of these investigations and the present state of knowledge in this field will now be considered in some detail.

### (1) *Technique for studying the absorption of blood constituents by the mammary gland*

The uptake of blood sugar by the active mammary gland of the cow as compared with that of the resting gland was studied by Kaufmann & Magne (1906), whose results have been subsequently interpreted as providing evidence in favour of the view that blood sugar is the precursor of lactose. It will later be seen that their technique was subject to a serious error. Assuming that the amount of blood sugar used by the tissues of the head for energy production was about equal to the amount required for similar purposes by the active mammary gland, Kaufmann & Magne considered that the sugar content of jugular blood could be taken as representing that of arterial blood minus the sugar utilized by the mammary gland for production of energy. They found experimentally that in the lactating cow the sugar content of jugular blood was higher than that of the blood leaving the mammary gland by way of the subcutaneous abdominal (mammary) vein, while in the non-lactating cow the sugar contents of the two bloods were about equal. It was considered that the difference in sugar content between the blood from the two veins represented the sugar used by the lactating mammary gland for purposes other than energy production. Blackwood & Stirling (1932*a*) have pointed out that Kaufmann & Magne were in error in that they neglected to consider the effect on the jugular blood composition of the loss of water due to salivation. This factor must be of considerable magnitude in studies on the cow since, according to Schalk & Amadon (1928), the ox secretes some 60 litres of saliva in 24 hr. Blackwood & Stirling, and later Blackwood & Wishart (1936), have put forward evidence that appreciable concentration of the blood coming from the head is thereby caused and hence that conclusions based on comparisons of the composition of jugular blood with that of mammary venous blood in the cow are suspect. On the other hand, there is evidence that the blood passing through the mammary gland usually undergoes practically no concentration nor dilution (Blackwood & Stirling, 1932*a*; Lintzel, 1934; Graham, Kay & McIntosh, 1936), though in cows which were

excited during sampling, Shaw & Petersen (1939) observed exchanges of fluid between blood and mammary gland of magnitudes sufficient to affect arterial-venous differences in non-diffusible blood constituents.

Modifications of the Kaufmann-Magne method have recently been used to study the uptake of sugar and other blood constituents by the mammary gland of the cow and goat, and it is not out of place briefly to consider here the value of such methods in lactation studies. In the first place, the considerations just discussed make it evident that the composition of mammary venous blood must be compared with that of arterial blood. Secondly, for each comparison, samples of blood from the two sources must be taken as nearly simultaneously as possible (in any case the arterial blood must never be taken before the venous sample) and with a minimum of disturbance to the experimental animal. The last condition is important since undue disturbance arising from blood sampling not only alters the composition of the blood, in particular with respect to the concentration of sugar, but also causes blood-volume changes in the mammary gland together with alterations in the arterial-venous differences (see Graham, Kay & McIntosh, 1936; Shaw & Petersen, 1939). The best modifications of the Kaufmann-Magne method hitherto devised for application to the cow are those described by Graham, Kay & McIntosh (1936) by which it is possible to obtain approximately simultaneous samples of bovine arterial blood (by puncture of the internal iliac artery through the rectal wall) and mammary venous blood, and by Maynard *et al.* (1937) who puncture the internal pudic artery through the vagina.

All studies of the uptake of blood constituents by the bovine mammary gland which have been carried out up to the present are, however, open to the following criticisms, which also apply to Lintzel's (1934) work on the goat. It is probable that some proportion of the blood constituents absorbed by the active mammary gland will pass into the lymph, and at present there is no known method of estimating the magnitude of this factor. A further, and possibly considerable (but see below) proportion of the substances abstracted from the blood by the mammary gland will be utilized by the latter for the production of energy. Without data regarding the energy requirements of the mammary gland in various states of activity it is impossible quantitatively to interpret the results of experiments made by the Kaufmann-Magne method. Up to the present the only estimate of the energy requirements of the active mammary gland is that of Graham, Houchin, Peterson & Turner (1938) who found that only about 10% of the energy received by the mammary gland from the blood is used for metabolic purposes.

Further, for a given rate of milk secretion, and hence of uptake of blood constituents by the active mammary gland, the observed arterial-venous difference for any milk precursor will depend on the rate of flow of blood through the gland. Therefore, arterial-venous differences cannot be quantitatively interpreted in the absence of independent data concerning the mammary circulation rate. Finally, it must be remembered that a considerable volume of blood leaves the mammary gland by vessels other than the abdominal subcutaneous veins, so that the

composition of blood taken from the latter may not be representative of that coming from the mammary gland as a whole.

Recently, Graham, Houchin & Turner (1937), Graham (1937) and Graham, Peterson, Houchin & Turner (1938), working with goats, have devised a technique to which some of the above criticisms do not apply. Each goat was prepared for experiment by the exteriorization of one of the carotid arteries, while the amputation of one half of the udder and the tying and section of the external pudic and perineal veins left one major outlet for the blood leaving the remaining mammary gland. Further, during some of his experiments, Graham (1937) measured the rate of flow of blood through the mammary vein by a thermostromuhr method.

### (2) *The precursors of lactose*

Early attempts to determine the origin of lactose have been well reviewed by Meigs (1922); for an account of them the reader is referred to Meigs's article.

Blackwood & Stirling (1932*b*) were the first to compare the sugar content of arterial blood with that of mammary venous blood, and in lactating cows they found that blood from the mammary vein contained, in various cases, from 5 to 16 mg. per 100 ml. less sugar than blood taken simultaneously from the radial artery. In the case of non-lactating cows the arterial-venous differences were usually smaller. Lintzel (1934), working with the lactating goat, showed that the level of sugar in blood from the left ventricle of the heart was, on the average, about 20 mg. per 100 ml. higher than blood from the mammary vein. Similarly, Graham, Jones & Kay (1936), using the blood-sampling technique described by Graham, Kay & McIntosh (1936), observed average arterial-venous differences during lactation in the cow amounting on the average to about 10 mg. sugar per 100 ml. They made the further important observation that the arterial-venous blood-sugar difference was positively correlated with the arterial blood-sugar level. Their results also suggested the existence of a positive correlation between the arterial-venous blood-sugar difference and the daily milk yield, which would indicate that the arterial blood sugar level may be a limiting factor in milk secretion. Shaw, Boyd & Petersen (1938), however, failed to observe any correlation between arterial-venous sugar differences and time since milking, blood-sugar level or milk yield. It is unfortunate that the three last-named investigations did not include studies of non-lactating animals, particularly since the data of Blackwood & Stirling (1932*b*) suggest that the arterial-venous blood-sugar difference may be appreciable in absence of milk secretion. The experiments just discussed, however, taken as a whole, show that considerable amounts of sugar are absorbed from the blood by the lactating mammary gland.

If the arterial-venous blood-sugar difference and the percentage of lactose present in the milk secreted during the experiment are known, the number of litres of blood necessary to provide the sugar for the synthesis of the lactose in one litre of milk can be calculated. From the quantity so derived and the daily milk yield of the experimental animal under consideration, an estimate of the mean rate of blood flow through the mammary gland can be made. In this way, Graham,

Jones & Kay (1936) calculated that some 500 litres of blood must pass through the mammary glands of their experimental cows to provide the sugar contained in 1 litre of milk, which implied a mammary circulation rate of nearly 12 litres a minute, while Lintzel's (1934) estimate for the lactating goat was 256 litres of blood to provide the sugar for each litre of milk. These calculations are, however, only valid if the mammary gland utilizes none of the sugar for energy production or purposes other than lactose synthesis, and if blood sugar is the only blood constituent from which lactose is formed. It is easy to see that if blood sugar is not the sole precursor of lactose a much lower circulation rate would suffice to provide the materials necessary for lactose synthesis. Direct measurements by Jung (1933) and Graham (1937) indicate a much lower mammary circulation rate than do the indirect calculations of Lintzel (1934) and Graham, Jones & Kay (1936), so that the possibility that other blood constituents besides sugar are converted into lactose must be considered. The results of sugar-balance experiments carried out on the mammary gland of the lactating goat by Graham (1937) point to the same conclusion. In these experiments the observed blood sugar uptake was insufficient to account for all the lactose secreted during the experimental period, even supposing that all the absorbed sugar was converted into lactose. Graham (1937) further observed that considerable quantities of lactic acid were absorbed from the blood by the lactating mammary gland. In two balance experiments in which uptake of considerable amounts of blood sugar, blood lactic acid and blood amino nitrogen by the active gland was observed, about 85% of the lactose produced in each experiment could be accounted for on the assumption that all the sugar and lactic acid were converted into lactose. If the amino-acid nitrogen absorbed during the experiment was calculated as the three-carbon-atom amino acid, alanine, the remaining lactose could be accounted for by assuming the conversion by the mammary gland of this amino acid into lactic acid and thence to lactose. It is quite possible that the active mammary gland is indeed capable of deaminating amino acids, since Graham, Houchin & Turner (1937) observed the production of considerable quantities of urea by the mammary gland of the lactating goat, and Shaw & Petersen (1938*a*) detected the presence of arginase in the active bovine udder. Shaw *et al.* (1938) confirmed the uptake of lactic acid by the active bovine udder but their arterial-venous differences for sugar and lactic acid were together sufficient to account for all of the milk lactose.

In summing up, it may be said that the results of investigations of the absorption of blood sugar by the lactating mammary gland are consistent with the view that blood sugar is a precursor of lactose, though the most recent work indicates that other blood constituents are also concerned in lactose synthesis. Further data on the energy requirements of the mammary gland are, however, urgently required since it is important to know how much of the absorbed sugar is utilized by the mammary gland for energy production.

There is a certain amount of additional evidence which points to a relationship between blood sugar and lactose. In general, procedures which diminish the level of blood sugar in the lactating animal tend also to produce a lowering of the lactose

content of milk, and there is reason for believing that the reverse is also true. In the cow, inanition (Gowen & Tobey, 1931 *a*) or treatment with insulin (Gowen & Tobey, 1931 *b*; Brown *et al.* 1936) or phlorizin (Gowen & Tobey, 1931 *b*) cause the lactose concentration in the milk to diminish and the blood sugar is lowered at the same time. Incidentally, Nitzescu (1933) observed in sheep that during a period of inanition the lactose content of the milk could be restored to the normal level by injections of the monosaccharides dextrose, fructose and galactose and the disaccharide maltose, but not with the disaccharides sucrose and lactose.

On the other hand, Whitnah *et al.* (1933) were able to produce temporary increases in the blood sugar concentration in the cow by pumping glucose solutions into the stomach, and these changes were always accompanied by temporary increases in the milk lactose concentration. Further, Bottomley *et al.* (1939) produced quite prolonged hyperglycaemia in goats by subcutaneous implantation of adrenalin tablets; the milk lactose content was found to increase at the same time. Thyroxine administration also increases the blood sugar level, and it is significant that the lactose concentration of the milk of the cow increases during hyperthyroidism (Jones, 1935).

The problem of the origin of lactose has been attacked by attempting to effect lactose synthesis *in vitro*. Isaac & Adler (1921), Svanberg (1930), Michlin & Lewitow (1934) and others have attempted to demonstrate the synthesis of lactose from various sugars in the presence of extracts or other preparations of lactating mammary tissue, but their claims are hardly convincing. Weinbach (1936) observed an increase in non-fermentable, reducing disaccharide when glucose was incubated with a powder prepared from dried mammary tissue. His data led him to postulate the existence of a non-reducing lactose precursor which breaks down to lactose in presence of glucose, a contention which requires further experimental support before it can be accepted.

The most satisfactory demonstration of lactose synthesis *in vitro* has been made by Grant (1935), who has shown that when thin slices of active mammary tissue are incubated with suitable physiological salt solutions containing glucose, lactose is synthesized at the expense of glucose. No appreciable lactose synthesis was observed when the glucose was replaced by fructose, mannose or galactose. Grant's results suggest that the mechanism of lactose synthesis is not the two-stage reaction $\alpha$-glucose$\rightleftharpoons\beta$-galactose; $\alpha$-glucose$+\beta$-galactose$\rightleftharpoons$lactose, since he found that in mixtures of glucose and galactose the extent of lactose synthesis corresponded merely to that expected from the glucose content alone. Petersen & Shaw (1937) isolated lactose from mixtures of glucose and lactic acid which had been incubated with macerated mammary gland tissue. In view of the fact that Grant was able to demonstrate lactose formation in the presence of glucose alone, this can hardly be taken as evidence that lactic acid is a precursor of lactose.

In view of the importance of phosphorylation in fermentation and intermediate muscle metabolism, the possibility that phosphoric esters may act as intermediates in lactose synthesis must not be overlooked. This has been suggested by Barrenscheen & Alders (1932), while Borst (1932) and Brenner (1932) have demonstrated

the presence of phosphoric esters in mammary gland tissue. Further, Folley & Kay (1935) have shown that mammary gland tissue is one of the most potent sources of phosphatase in the mammalian body. Grant (1936), however, was unable to demonstrate increased lactose synthesis by mammary tissue slices in presence of a variety of hexose monophosphates or of phosphoglycerate. Nevertheless, the results of Aten & Hevesy (1938), who used radioactive phosphorus in a lactation study, indicate that rapid breakdown and synthesis of phosphoric esters occur in the active mammary gland.

We thus see that experimental approach from various angles has produced a considerable amount of evidence that sugar absorbed from the blood is converted into lactose by the lactating mammary gland. No evidence contrary to this hypothesis exists. At the present time, however, little or nothing is known of the mechanism of the synthesis beyond the probability that perhaps lactic acid and other blood constituents enter into the reaction, and this question is suggested as a fruitful field of study.

### (3) *The precursors of milk fat*

Various possibilities as to the origin of the milk fat, which has a composition found nowhere else in nature, present themselves. The more probable of them are listed hereunder. Milk fat may be formed (*a*) by chemical transformation of the neutral fat of the blood, (*b*) from the blood phospholipins, (*c*) from glycerol derived from blood sugar and fatty acids from the cholesterol esters of the blood, (*d*) from blood sugar alone by an extensive series of chemical reactions, (*e*) by some combination of two or more of the foregoing. *A priori* the most probable hypotheses of the mode of secretion of milk fat are the two first named.

The theory that milk fat is formed in the mammary gland from the blood phospholipins was put forward by Meigs *et al.* (1919), and later supported by Nikitin (1935) on the basis of results obtained by the original method of Kaufmann & Magne. Meigs *et al.* found that, in the lactating cow, blood from the subcutaneous abdominal vein contained less phospholipin phosphorus than jugular blood, while in dry cows the differences in the lipin phosphorus content of blood from the two sources were not so pronounced. They further pointed out that if, as their results appeared to them to suggest, the blood phospholipins are the precursors of milk fat, the mammary gland would absorb from the blood more than sufficient phosphorus as lipin phosphorus to provide the phosphorus of milk. They found that the mammary venous blood in their lactating cows, in fact, contained more inorganic phosphorus than the jugular blood, which appeared to them to prove that the phosphorus derived from phospholipins, absorbed by the mammary gland in excess of that necessary to provide the phosphorus of the milk, was carried away from the mammary gland by the efferent blood.

As we have seen, the assumption that the composition of jugular blood may be taken, without serious error, to be representative of that of arterial blood is unjustified. The question has since been re-examined by Blackwood (1934), by Graham, Jones & Kay (1936), and by Maynard *et al.* (1938), working with the lactating cow,

and by Lintzel (1934), working with the lactating goat, in each case the composition of mammary venous blood being compared with that of arterial blood. The results of all three investigations agree in showing that the hypothesis of the origin of milk fat from blood phospholipins is untenable, since in no case could any significant absorption of phospholipin phosphorus by the active mammary gland be demonstrated. Further, these workers, with the exception of Maynard *et al.* (1938), have shown that there is a definite decrease in the concentration of inorganic phosphate in the blood during its passage through the lactating mammary gland. Since there has been shown to be very little consistent variation in the concentrations of other "phosphorus fractions" as between arterial and mammary venous blood in lactating animals, it may be concluded that the phosphorus of milk largely comes from the inorganic phosphorus of the blood.

In agreement with the above-mentioned work, the results of Aten & Hevesy (1938) using radioactive phosphorus, demonstrate without a doubt that blood phosphatides do not enter into the formation of milk fat, nor does the inorganic phosphorus of the milk arise from blood phosphatide phosphorus.

The theory of the origin of milk fat from the neutral fat of the blood was supported many years ago by the perfusion experiments of Foà (1912) who found that when the surviving sheep's udder was perfused with Ringer's solution in which olive oil or triolein had been emulsified, the gland secreted a watery fluid containing globules of fat, the iodine number of which was lower than that of the fat in the perfusion fluid. This work was criticized by Meigs (1922) on the ground that, since the gland was reported by Foà to have become oedematous during the course of the experiment, the permeability of the secreting cells would have thereby become altered to such an extent as to invalidate the results. The results of Petersen *et al.* (1929*b*) differed from those of Foà in that they found that the secretion resulting from perfusion of the udders of cows had an abnormally low fat content, while perfusion of such udders with an emulsion of corn oil failed to increase the fat content of the resulting secretions. When an emulsion of corn oil coloured with Sudan III was used, though the fat in the gland secretion contained no colour, about 3% of the dye was found in the gland tissue which indicates that the mammary gland is capable of absorbing neutral fat. Recent experiments, however, lend considerable support to the theory that milk fat arises from the blood triglycerides. Lintzel (1934) was able to obtain indirect evidence of a diminution in the blood triglycerides during the passage of the blood through the mammary gland of the goat, though he could obtain no evidence of the utilization by the mammary gland of cholesterol ester fatty acids. Similar results with the cow were reported by Graham, Jones & Kay (1936) though, as these workers point out, their results did not definitely exclude the possibility that some of the fatty acids of milk fat may come from the cholesterol esters of the blood. The experiments of Maynard *et al.* (1938) also indicate that milk fat is formed from the neutral fat of the blood, since they show that though the total lipoids of the blood decrease in passing through the mammary gland, there appears to be no decrease in phospholipins nor in cholesterol esters (see also Maynard *et al.* 1937). Further support to the

theory of the origin of milk fat from the blood triglycerides is afforded by the experiments of Aylward *et al.* (1937), who found that the lipaemia caused by feeding iodized fat to lactating cows was accompanied by the secretion of iodized fat in the milk. They found that a relatively small proportion of the iodized fatty acids entered the phospholipins of the blood, so that the major proportion of the iodized fatty acids appearing in the milk must have been carried to the mammary gland as glycerides or cholesterol esters.

The present position may be summarized by saying that there is no evidence that any appreciable fraction of the fatty acids of milk fat comes from the phospholipins of the blood, while there is a certain amount of evidence that the major portion of them arises by chemical transformation from the fatty acids of the blood neutral fats. Before the question of the origin of milk fat can, however, be regarded as settled it will be necessary to know what proportion of the blood fat absorbed by the mammary gland is oxidized for energy production. Shaw & Petersen (1938*b*) believe that their data show that a portion of the fatty acids from the neutral fat absorbed from the blood by the mammary gland is oxidized, thus giving rise to the lower fatty acids of milk fat.

### (4) *The precursors of caseinogen*

The two most probable theories as to the precursors of caseinogen are (*a*) that it is synthesized in the mammary gland from blood amino acids, the phosphorus being provided by the inorganic phosphorus of the blood, or (*b*) that it arises from the proteins of the blood plasma which may be first broken down in the mammary gland to still relatively complex fragments from which the caseinogen molecule is synthesized. Since amino acids are present in the blood in relatively small concentration it is to be expected, if the first theory were true, that the modified Kaufmann and Magne method would reveal an appreciable uptake of amino nitrogen by the mammary gland. If, however, the second hypothesis were true a diminution in the protein nitrogen of the blood would occur during its passage through the mammary gland.

Cary (1920), comparing the composition of samples of jugular and mammary venous blood taken from lactating and dry cows, found that in the latter the amino-acid nitrogen contents of blood and plasma from both sources were approximately equal, while with lactating cows the blood and plasma from the mammary vein contained less amino-acid nitrogen than that from the jugular vein. Blackwood (1932) for the cow and Lintzel (1934) for the goat, comparing arterial blood with mammary venous blood and using improved methods of estimating amino nitrogen, have since shown that there is an appreciable absorption of amino-acid nitrogen from the blood plasma by the lactating mammary gland which is consistent with the first hypothesis stated above.

Graham (1937), however, concluded from the results of his balance experiments already referred to that the uptake of blood amino nitrogen by the mammary gland of the lactating goat was insufficient to provide the nitrogen of the milk secreted during the experiments. In subsequent experiments carried out on lactating goats,

Graham, Peterson, Houchin & Turner (1938) showed that in addition to amino-acid nitrogen, the active mammary gland absorbed from the blood considerable quantities of non-protein nitrogenous substances and also of globulin.

The arterial-venous blood amino nitrogen differences observed by Shaw & Petersen (1938*c*) were also insufficient to account for more than some 35% of the milk protein, that is, assuming that blood volume to milk volume ratios for fat, carbohydrate (sugar + lactic acid) and calcium, calculated from arterial-venous differences as explained above, which agreed fairly well among themselves, represent the true state of affairs. Shaw & Petersen (1938*c*) also found that the active gland absorbed very little uric acid, creatine and creatinine. It would therefore appear that, while it is possible that blood amino acids are utilized for synthesis of caseinogen in the mammary gland, an important source of milk protein is some fraction of the plasma proteins which is estimated as globulin. The phosphorus of caseinogen appears to come from the inorganic phosphate of the blood (Aten & Hevesy, 1938).

### (5) *Enzymes of the mammary gland*

Studies of the nature and properties of the enzyme systems present in living tissues have often yielded valuable information concerning the chemical changes which make possible their characteristic functions. Unfortunately such studies have hitherto not been extensively applied to the mammary gland. If our present knowledge of the enzyme equipment of the alveolar cells of the mammary gland were extended, as it could not fail to be if modern developments in enzyme chemistry were applied to mammary gland studies, it is certain that much more would be known than at present of the way in which milk constituents are synthesized. The brevity of the following account of existing information concerning the enzymes of the mammary gland will, it is hoped, indicate the urgent need for further research in this field.

Of mammary gland proteolytic enzymes, very little is known. Grimmer (1913) has studied the proteolytic enzymes which cause autolysis of excised mammary tissue and identified some of the amino acids formed during autolysis. Tateyama (1925) reported the presence in extracts of human mammary tissue, both lactating and resting, of a peptidase which would hydrolyse glycyl-tryptophane. Arginase has been detected in the active mammary gland by Shaw & Petersen (1938*a*).

As regards lipases, the available evidence suggests that mammary tissue is poor in lipolytic activity. Various workers have detected enzymes which hydrolyse butyric acid esters. Of these Tateyama (1925) found that lactating mammary tissue was richer than resting tissue in esterase, and Virtanen (1924) gave the optimum *p*H as 8·7 for hydrolysis of ethyl butyrate by glycerol extracts of active mammary gland.

Amylases are present in lactating and also resting mammary tissue according to Grimmer (1913) and Tateyama (1925), who reported that lactating tissue was more active than resting tissue. The presence of a maltase (optimum activity at *p*H 6·9) in glycerol extracts of the gland was reported by Kleiner & Tauber (1932).

Purine enzymes in the mammary gland have been studied by Michlin & Ryżowa (1934).

The most closely studied enzyme of mammary tissue is the phosphatase which hydrolyses mono-esters of phosphoric acid optimally at *p*H 9–10. Clear evidence of its presence in mammary tissue was first obtained by Kay (1925). In the same year Tateyama observed that mammary gland extracts contained an enzyme which would dephosphorylate nucleic acid. Later, Borst (1932) confirmed the presence of a phosphatase in mammary gland tissue. Folley & Kay (1935) have studied the properties of the phosphatase of guinea-pig mammary gland and shown that as regards *p*H optimum, Michaelis constant, the effect of substrate composition on *p*H optimum and Michaelis constant, and finally, activation by magnesium, it is identical with the alkaline phosphatase of the kidney and is, therefore, on the basis of the phosphatase classification proposed by Folley & Kay (1936*a*), to be classed as a phosphomonoesterase $A_1$. Of great interest is their finding that in the guinea-pig, and presumably in other species also, the phosphatase activity of the mammary gland is remarkably high, of the same order in fact as that of whole kidney tissue. The mammary gland is, therefore, one of the three or four most potent sources of the phosphomonoesterase $A_1$ in the mammalian body.

In a preceding section the presence of a phosphatase in milk was mentioned. This enzyme is also found in blood plasma but in lower concentration than in milk. Since, as far as is known, the mammary gland does not possess a mechanism analogous to that of the kidney for concentrating and excreting blood constituents it is probable that the milk phosphatase does not arise directly from the blood plasma, but that it is an excretory product of the mammary gland cells (see Folley & White, 1936; Folley & Kay, 1936*b*). Cow's milk contains quite considerable amounts of phosphatase and hence during lactation the bovine mammary gland must lose daily a large amount of this enzyme. The mammary gland of the cow thus appears to be the site of rapid and continuous synthesis of phosphatase during lactation.

## V. SUMMARY

1. In most species oestrogens mainly stimulate mammary duct growth. In the guinea-pig and goat they promote complete alveolar development. The mouse is the only animal in which treatment with progesterone has been found to promote mammary duct growth. In other species simultaneous treatment with oestrogens and progesterone is necessary for complete alveolar development. It is difficult to say whether or not oestrogens *per se* can promote alveolar development, because the adrenal cortex produces progesterone.

2. Androgens cause duct and alveolar growth in some species.

3. Some now believe that, under the influence of oestrogen, the anterior pituitary produces a "mammogenic" hormone which promotes mammary duct growth.

4. The presence of the anterior pituitary is essential for the initiation and maintenance of lactation. The anterior lobe is believed to secrete a specific lactogenic hormone (prolactin), but other anterior lobe hormones probably participate in the control of normal lactation.

5. Oestrogens may inhibit lactation during pregnancy, but it is probable that the pregnant uterus is also concerned in the inhibition.

6. The adrenal-cortical hormone is necessary for lactation, since adrenalectomized animals do not lactate, and, further, prolactin will not initiate or maintain lactation in hypophysectomized animals unless corticosterone or adrenotrophic hormone is also given.

7. The thyroid gland is essential for normal lactation: thyroxine stimulates milk secretion in the cow.

8. Posterior pituitary extracts cause emptying of the bovine udder but do not affect the formation of milk.

9. Oestrogen treatment enriches milk in fatty and non-fatty solids, and so does thyroxine treatment. Repeated injections of certain prolactin extracts increase the fat content of cow's milk; certain other prolactin preparations increase the lactose content.

10. No true secretory nerves innervate the mammary gland. Lactation is disturbed in the cat if the sympathetic chains are extirpated.

11. The extraction of milk from the bovine udder depends on the reflex erection of udder tissues caused by stimulation of the teat, which also appears to cause the release of a lactogenic hormone or hormones from the hypophysis.

12. The mammary gland synthesizes lactose from blood glucose and also, probably, lactic acid. It is also possible that blood amino acids are de-aminated by the mammary gland and used for lactose synthesis.

13. The precursor of milk fat is the neutral fat of the blood. The mammary gland probably oxidizes long-chained fatty acids to the short-chained acids of milk fat.

14. It is improbable that milk protein arises from blood amino acids. Plasma proteins are probably utilized for the synthesis of caseinogen, the phosphorus coming from the blood inorganic phosphate.

15. Various enzymes have been detected in active mammary tissue, which is a particularly rich source of the alkaline phosphomonoesterase. Changes in the secretory function of the mammary gland are usually accompanied by striking changes in the phosphomonoesterase content of milk.

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